

What future for postwar Bosnia?

It is not too soon for the rest of Europe to begin preparing for the aftermath of the tragic warfare in ex-Yugoslavia; on the contrary, doing nothing now is a recipe for permanent calamity.

THREE years of warfare in ex-Yugoslavia have left a scar on the world's civility, and will almost certainly cause further harm before the fighting ends. For those who are most tragically afflicted, the people who live in the once-federated states and in Bosnia and Herzegovina in particular, the scars will not be healed for decades, until recollections of those who have been killed or maimed have faded. The worst outcome, not improbable, is that troubles like those besetting Northern Ireland for the past quarter of a century will become endemic in much of what used to be Yugoslavia. If and when peace is declared, there will be ample supplies and sources of munitions to give people the illusion that they can settle old scores without making things even worse. The temptation to follow that course will be strengthened by the way in which the community (at least in Bosnia and Herzegovina) has been confirmed in its three-way division by recent horrors.

The rest of Europe could not live with such a state of affairs without its own security being undermined. Already the reputation of the European Union or EU (and of its designated but still-theoretical military arm, the Western European Union) has been damaged by the failures of three years ago. The decision, at the insistence of Germany, to recognize Slovenia as an independent state made the break-up of the federation irreparable, but Western Europe's most prosperous states also too eagerly embraced the correct legal doctrine that one state cannot intervene in the domestic affairs of another as an excuse for doing very little. But short of military intervention, there is much that could have been done by political means (carrots as well as sticks) to make the rewards of inter-communal violence less attractive.

The dangers for the rest of Europe of chronic strife in the Balkans are easily foretold. Experience elsewhere has shown how local divisions are reflected internationally. At least some of the tension between Russia and the United States apparent in the past few weeks is, for example, directly attributable to a single air strike around Gorazde. (That the strike was ordered by the Secretary-General of the United Nations, an Egyptian, on the advice of his Japanese deputy in the field, and carried out by NATO aircraft in accordance with a resolution of the Security Council is relevant, but seems not to be so regarded.) Even if there is a formal settlement in ex-Yugoslavia, the risk that there will follow a trial of strength by proxy between backers of the three sub-communities must be very high.

The economic and social damage that has and will be done in ex-Yugoslavia must also be reckoned with. For a country whose exports consisted largely of the hospitality for which tourists paid, patterns established in the past three years of going elsewhere will persist. But who then, and with what resources, will rebuild those damaged houses and restore damaged farms to produce work? Especially if the United Nations embargo is itself maintained? There is all too real a danger that ex-Yugoslavia, already pauperized by the inflation that seems inseparable from warfare, will become a pauperized patch of poverty on the Southern rim of what is Western Europe. That would be insupportable.

But what can be done? The temptation to believe that it is too soon to be thinking constructively should be suppressed. Culturally and economically, ex-Yugoslavia is as much a part of the EU as, say, the Baltic States or the Slavic states of Central Europe, with all of which the EU is holding preliminary talks on eventual membership. It would be a carrot of sorts, and perhaps a powerful one, if the EU were to say that it seeks a way of dealing with ex-Yugoslavia and its parts, jointly or severally, on the same footing. More immediately, it would help if the rest of Europe followed the University of Warsaw in offering to help educate the cohorts of potential students who will otherwise be denied an education in ex-Yugoslavia. Both these steps, and others, would cost money, but probably not as much as the Balkans will otherwise cost Western Europe. □

Instant legislation

A British Cabinet minister has been helping to highjack legislation for populist purposes.

BRITAIN, which has traditionally boasted of its way of conducting parliamentary business in what it likes to call the 'Mother of Parliaments' (the House of Commons), appears to be falling apart in that connection as well as others. Last week, Mrs Virginia Bottomley MP, who is also the Secretary of State for Health in the British government, assisted in a plot to tag onto a much debated piece of legislation a populist and ill-considered prohibition of the use, in the treatment of infertility by *in vitro* fertilization (IVF), of ova from aborted fetuses. The merits of the prohibition apart, the case has all the hallmarks of the procedures in the US Congress by which



pork-barrel amendments are irrelevantly tagged onto legislation with quite different purposes.

British parliamentarians, who profess distaste for the way that US congressmen ambush unconnected legislation to serve the needs of their constituents, should reflect carefully on what was done last week. Since 1990, both research and medical procedures using human embryos have been strictly regulated by a statutory authority, the Human Fertilization and Embryology Authority. The only complaints which there have been of the authority is of its tendency to officiousness. Earlier this year the authority put out a consultation paper which fairly outlined the pros and cons of using ova taken from aborted fetuses as well as from stillborn children and women who have died. The consultation period (extended because of the volume of comment) is due to end in July.

Evidently Mrs Jill Knight MP, a long-serving member of the House of Commons, cannot wait for that process to be complete. Having failed, earlier this year, to win parliamentary time for a bill to ban the use of fetal ova in IVF, she had sought to tag a similar provision onto this year's Criminal Justice and Public Order Bill now making its way through the British parliament. That is fair enough. The surprise is that Bottomley should have announced during the proceedings that she would be voting for the amendment. That is a bad business, and a serious dereliction of duty by a government minister, whatever her personal opinion about the rights and wrongs of the use of fetal ova.

This is why. First, there is no prospect that the HFEA will sanction the use of fetal ova in IVF while its consultation is still under way. Second, it is unthinkable that such proposals would be approved while it is not known why the number of ova in a female fetus is ordinarily reduced by more than four orders of magnitude by the time of ovulation; the possibility that the discarded ova are genetically impaired would first have to be excluded. Third, the consultation has itself raised an important issue that would have to be decided before anything could be done; existing British guidelines on the use of fetal tissue allow consent to be given by the mother alone and require that the purpose should not be specified, while the law under which HFEA operates requires that IVF procedures should be genetically transparent. Fourth, there is no need of legislation: failure to comply with the HFEA's licence terms is already a criminal offence.

Bottomley knows that, and should have said so. Her failure to do so is bound to undermine the authority of the HFEA, whose consultation process has been made to seem largely irrelevant. But the terms of her endorsement of the amendment are also alarming: Mrs Knight's advocacy of her cause had "hit a nerve". Yet Mrs Bottomley's own interest as a minister is that such matters should not be decided emotively. The best hope now is that the House of Lords, which will have another chance to say what it thinks of the bill, will throw out the amendment on the grounds that it is superfluous. Meanwhile, the whole shabby incident is yet another reminder that Britain needs better parliamentary procedures. □

How to lose confidence

The scandal over fraudulent data in a breast-cancer study undermines trust in the research establishment.

LAST month the *Chicago Tribune* reported that some participants in a major study of lumpectomy plus radiation versus total mastectomy for early breast cancer did not meet the study's protocol. And worse: research physicians coordinating the study at the University of Pittsburgh as well as its overseers at the National Cancer Institute (NCI) had known about the aberrations since 1991. The Office of Research Integrity also knew about the faulty data, as did officials at the Food and Drug Administration. But no one said a word.

Roger Poisson, a breast-cancer surgeon at St Luc's Hospital in Montreal, falsified the records of at least a hundred women in the surgical study and also submitted falsified data in that part of it meant to evaluate the drug tamoxifen as adjuvant therapy. Poisson admits enrolling women who were technically ineligible, in some cases because they had not made a decision about surgery within 30 days of diagnosis as specified in the research protocol. More than 450 hospitals, caring for some 5,000 women, have contributed data. A preliminary analysis, excluding Poisson's data, confirms that lumpectomy is every bit as effective as more radical total mastectomy in treating early breast cancer.

But that is not the point. Every day, hundreds of women all over the world make decisions based on the conclusions of this study, which until last month was headed by Bernard Fisher, a Pittsburgh surgeon who has devoted more than 20 years changing the long-standing belief in radical mastectomy. And now those women have lost confidence in the US government. It does not matter that statistical analysis says the data hold up. What matters is that everyone who bears any responsibility for the study knew about the aberrant data and no one spoke up, probably from a well-intentioned but paternalistic instinct not to upset women needlessly.

What should Fisher and the others have done? The answer is easy. As soon as they learned that Poisson had failed to follow the protocol, they should have excluded him from the study with a public announcement to that effect. They could have added that the aberrant data in no way changed the presumption in favour of lumpectomy, but that a scientist who tampers with the data, however benignly, will not be tolerated. They should have known that the fraud would eventually come out — it always does — and that newspaper accounts of a cover-up would do far more damage to the public trust than a clear statement about a single surgeon who thought he could bend the rules and get away with it.

Last week, Samuel Broder, head of the NCI, and Harold Varmus, the new director of the National Institutes of Health, found themselves in the embarrassing position of apologizing to Congress for their silence (see page 679). At a hearing before Representative John Dingell (Democrat, Michigan), they tried to put the blame on Fisher. But in this case, there is plenty of blame to go around and everyone (except Varmus, who is too new) should accept his share. □

France backs down on radical reform after resistance from researchers

Paris. France's conservative government has backed away from ambitious plans to transform the country's research system following its six-month national 'consultation' on research, which culminated this week with a colloquium in Paris attended by prime minister Edouard Balladur.

When the coalition government came to power last year, some of its more conservative members pushed for the dismantling of public research organizations and a reinforcement of the university system. They also urged the government to reorientate research heavily towards wealth creation.

During the consultation, however, many scientists told the government that this was unrealistic. Last week saw the release of a "manifesto for research", signed by over 1,200 leading researchers, warning that a radical reorganization of the research system would provoke harsh resistance.

Speaking at the national colloquium, François Fillon, the minister for higher education and research, said that the government would not dismantle the research organizations. "The ideological debate about killing the CNRS [Centre National de la Recherche Scientifique] is now dead", he told the meeting.

Fillon said that during the consultation he did not hear many voices raised against the research organization. Moreover, he needs the consultation to succeed following his aborted effort to reform the universities (see *Nature* 364, 472; 1993), and after successive climbdowns — on a restructuring of Air France and a low wage scheme for young people — the government cannot afford to lose a further confrontation.

Fillon says he heard much criticism of the rigidity of the French research system. But even here, he has conceded to the demands of researchers that reforms of such problems requires a continued growth in the research budget, even at a low rate.

Among the reforms that Fillon proposes are the creation of a single professional status for researchers that would allow them to move freely between the research organizations, the universities and industry.

He also wants a national committee to establish national priorities in research. But he accepts that the research community itself is best placed to determine these. While claiming that excessive government control would diminish the diversity of research and innovation, he says that managing research on the basis of market needs is based on an

obsolete linear model of innovation, and would be an "error".

Fillon admits that, despite the national consultation, he will have fewer firm recommendations than he expected to put to a debate on science planned for the National Assembly in June, an occasion which the



Fillon: keen to avoid conflict.

government plans to use to launch a new national strategy for research.

Some observers claim that the national colloquium's failure to propose many policy changes is a sign of good sense. "Fillon has learnt that there is no sense in declaring war [on the research community]", says one government official. Indeed, the minister diplomatically welcomed last week's manifesto as a "constructive, if loud, contribution" to the consultation.

The manifesto was signed by 33 of the 40 members of the national committee of the CNRS, as well as all seven heads of its scientific departments and almost all its scientific board. It was also signed by six heads of scientific boards of the biomedical research organization INSERM, and 30 members of the national university committee.

Its basic message was that the government should think twice before radically reforming the research system. Henri-Edouard Audier, a chemist at the Ecole Polytechnique who organized the manifesto, says that it was intended as a form of self defence.

The lack of conclusions from the consultation has also reassured many researchers, who considered that the process had been rushed. They were also concerned that neither CNRS nor INSERM were represented on the committee which drafted its agenda.

Another major concession from Fillon is a decision to seek an immediate solution to the problem of scientific recruitment. Over half of all French researchers will retire by 2005, and Audier has called on the government to begin recruiting now in order to preserve a sensible age pyramid.

For his part, Balladur confirmed that he considered research a national priority. But the many who expected him to focus on the value of research in boosting competitiveness were surprised when he said that the main purpose of a strong science base was to provide France with political and cultural influence.

Declan Butler

Genome research consortium backed

London. A group of top British geneticists has suggested the setting up of a consortium of the main academic groups in the field, and involving both research councils and pharmaceutical and biotechnology companies, to develop the commercial applications of genome research.

In a report commissioned by the Office of Science and Technology and published in London on Tuesday, the group also recommends the creation of a mouse genome centre, to include expertise in genetic and physical mapping, and in positional cloning.

It emphasizes that Britain's strong clinical base forms an ideal platform for the successful exploitation of genome research. But it warns that the opportunity could be jeopardized if this base is damaged by moves to place the National Health Service on a more commercial footing.

The report was commissioned last year as one of the first acts of a newly-created Advisory Committee on Human Genome Research, set up by the government to suggest ways of building on the strengths of genome research in the UK and securing its potential benefits for health-care and for British industry.

Drawn up by an expert working group

chaired by Kay Davies, director of the Medical Research Council's (MRC's) new Clinical Sciences Centre at the Royal Hammersmith Postgraduate Hospital in London, the report focuses attention on two particular aspects of genome research: the need to improve the services provided by genome data bases, and ways of increasing the effectiveness with which research results are used by the private sector.

"Two things are wrong with current databases," says Davies. "First, many scientists do not have access to a decent database; and second, industry does not get access to the information as fast as it would like."

The new consortium is suggested by the working group as one way of encouraging the exploitation of genome research. It suggests that, like a small company, the consortium would have its own laboratories where much of the work would take place.

According to members of the working group, one potential co-ordinator for the new consortium would be the MRC's Human Genome Mapping Project Resource Centre. This is shortly to move to Hinxton Park in Cambridge, where it will be adjacent both to the Sanger Centre and the new European Bioinformatics Institute. □

Land feud holds up work on ESO's telescope in Chile

Munich. Building work on Mount Paranal in northern Chile, where the European Southern Observatory (ESO) plans to install the world's largest telescope, has started again after a three-week enforced stoppage, which has cost millions of marks and prompted ESO to look around for a new site.

A local court at Taltal, near Paranal, last Friday (15 April) withdrew an injunction against the construction company Skanska-Belfi which had been brought by a Chilean family claiming ownership of the land. But the family remains determined to continue its fight through the courts.

The dispute is a blow to the construction of the Very Large Telescope (VLT), a series of four 8.2-m optical dishes estimated to cost DM463 million (US\$275 million) which has already had its completion date put back because of complaints from ESO's eight member states that its costs had grown too high (see *Nature* 367, 4; 1994).

Mount Paranal was donated to ESO by the Chilean government in 1988 after a search into the ownership convinced it that the site was the government's to give. The government also guaranteed ESO immunity from local law, as is normal for an intergovernmental organization.

But the Latorre family, which claims to be the direct descendants of an admiral who was given the land in the late nineteenth century in recognition of acts of heroism in

four contradictory judgements within days of each other. A final decision from the full court is expected within a few weeks.

Meanwhile, the Latorre family successfully applied last month for an injunction to stop building work while the case was being resolved. ESO's director, Riccardo Giacconi, says that delays to the project, which he reckons to have cost millions of marks in wages and other costs, combined with uncertainty over the outcome of the Latorres' claim, have left him no choice but to look around for a new site, possibly outside Chile.

This is despite the fact that construction is already quite advanced; the top of the mountain has been levelled and concrete foundations are about to be poured. If the VLT has to be resited, Giacconi is optimistic that ESO could reclaim the money already invested in the site through international courts. But he is reluctant to take such action. "It wouldn't get the telescope built," he says.

But the issue of substance — the true ownership of the land — has not yet been tackled by the government, whose general slowness to react has been a major point of conflict with ESO. On 15 April, ESO officials met government officials to try to persuade them to take steps to secure ESO's future at Paranal. The government is now reported to be ready to challenge the Latorre family's claim to the land.

Giacconi, who claims that the ESO has become the victim of attempts by lawyers to win compensation from the government, feels that the Chilean government has been negligent in defending ESO's interests.

The affair has erupted just as ESO was concluding negotiations over various long-standing disputes with Chilean scientists and support staff, relationships with whom have been poor for the past decade.

Tensions began to ease last year with the appointment of Giacconi, who took immediate steps to resolve two particular disputes (see *Nature* 363, 384; 1993). One concerns a request from Chilean astronomers for guaranteed viewing time on the VLT; this had previously been rejected but Giacconi is now offering 10 per cent of the available time.

The other dispute has been over the right to collective wage bargaining. ESO does not recognize local trade unions, but Giacconi has proposed mechanisms that would both allow collective bargaining and establish arbitration for disputes. Negotiations over both topics are in abeyance until the dispute about land ownership is settled. A similar problem at La Silla, a site also 'donated' by the Chilean government where ESO now has 15 telescopes, was settled in 1964 when ESO purchased the land from those who claimed to own it.

Alison Abbott

UK parliament passes surprise ban on fetal embryos in IVF

London. Britain's health minister, Mrs Virginia Bottomley, has jumped the gun on a top-level advisory committee by agreeing to support a parliamentary move to ban the use of embryos from aborted fetuses in fertility treatment.

Earlier this year, the Human Fertilization and Embryo Authority (HFEA), which is responsible for regulating both research on and the application of fertilization treatment, published a consultation document asking for comments on the use of fetal embryos. This followed reports that the technique is already being studied in animals — and in humans in other parts of the world.

The consultation period ends in July, when the HFEA is due to draw up its recommendations to the government. Last week, however, Dame Jill Knight, Conservative member of parliament (MP) for Birmingham-Edgbaston, put forward an amendment to the Criminal Justice Bill in the House of Commons specifying that "female germ cells" taken from an embryo or a fetus should not be used "for the purposes of providing fertility services for any woman".

Despite protests from opposition members of parliament, one of whom described the decision to debate the issue before the HFEA consultation period has finished as "the worst way of legislating and of using Parliament", the resolution was adopted.

The HFEA issued a statement saying that it was prepared to accept parliament's decisions, but that consultation will continue as planned. HFEA officials pointed out that Knight's resolution is restricted to the use of embryos in treatment, and does not refer explicitly to the conduct of research.

MPs were given a free vote, which excused them from voting along strict party lines. But Bottomley expressed her support for the motion, adding that officials from her department had helped Knight draw up the wording of the amendment. The action has come in for sharp criticism from scientists in the field. Several claim that, whatever Bottomley's feelings on the matter, she should have waited for the HFEA report before making them known — particularly as it is likely to be several years before the techniques are ready for the safe application to humans.

"I am very worried about people who are misinformed producing an ill-drafted amendment in this way," says Roger Gosden of the University of Edinburgh, who works with embryos from animal fetuses. He argues that the current language could be used to "bring down the curtain on research on birth defects and miscarriages". Opponents of the amendment hope that its impact can be blunted by further amendments when it is debated in the House of Lords.

David Dickson



a war with Peru, insists that it retains the title to the property.

Demanding the departure of ESO from the mountain, the family took both the observatory and the government to another local court, in Antofagasta, last May. But rather than ruling on this issue, the court questioned the immunity of ESO from any future ruling it might make.

ESO challenged this decision, setting up a chain of appeals and counter-appeals that ended up in the higher supreme court. Two weeks ago, different court members issued

Biologists out of Africa over rhino dispute

Washington. Two US conservation biologists have been forced to abandon their research into the effects of removing the horns of rhinos in Namibia, after government officials took exception to their preliminary conclusions pointing out the dangers of such a procedure.

Joel Berger and Carol Cunningham of the University of Nevada at Reno left the country only days after an article that they had written appeared in the 4 March issue of the journal *Science*. The article argues that dehorning black rhinos may leave the animals unable to protect their young from predators.

Berger and Cunningham — a married couple who have carried out extensive studies of the ecology and conservation of bison — say they had to leave Namibia when the government refused to renew their authorization to conduct research, three years into a four-year programme.

But in a statement issued on behalf of Namibia's environment ministry, Malan Lindeque, chief ecologist at the Etosha National Park where much of the research was conducted, denies that the pair had been expelled, although he concedes that Namibia was dissatisfied with their work.

In Namibia, dehorning is carried out by government conservation agencies in order to make dehorned animals unattractive to poachers. The government keeps the valuable rhino horn, which it hopes to sell abroad if a current ban on such trade is lifted.

Berger and Cunningham started looking at the effects of this policy in 1991 with a \$130,000 grant from the National Science Foundation, as well as support from various other bodies in the US and Sweden. Their early results indicated that the calves of dehorned rhino were likely to die if predators, such as hyenas, were present. The

Science article argues that "dehorning appears imprudent unless dangerous carnivores are removed".

Berger says Namibian officials were kept informed of the work through progress reports, including one in July 1993 that contained key language "almost identical to the

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Berger (right) claims that anti-poaching procedures leave young rhinos defenceless.

Science paper". But he says they were upset by an article published in the journal *Conservation Biology* last December, and that "the *Science* paper was the straw that broke the camel's back".

Berger and Cunningham returned to Namibia from a period in the United States on 1 March, and gave officials proofs of the *Science* article. They were asked to submit a new research proposal for the coming year, which they did.

But they were told that the new proposal would need to be reconsidered from scratch, and that, according to Berger, "we were free to stay in Namibia as tourists but not to continue research". Given growing hostility to their presence from some biologists and non-government organizations, the pair returned to the United States on 20 March.

Berger describes the Namibian response to the research findings as "hysterical" and claims that one official said the underlying reason was the money at stake in conducting the dehorning programme and in selling the horns. But Lindeque says that Berger and Cunningham have "grossly overreacted" and displayed "hypersensitivity to criticism" in leaving the country. "We have remained willing to resolve [the situation] in Namibia," he says.

Lindeque adds that the country's hospitality to foreign researchers should be reciprocated by a regard for local customs and concerns. "Our country and our way of managing it is more than just another study area or project for a foreign scientist, to be coldly dissected in foreign scientific journals," he says.

He argues that the two researchers showed no sensitivity to issues of concern to Namibians. "They chose to communicate with us by way of American scientific journals, claiming that pressure from grant sources to publish quickly superseded the courtesy of obtaining comment from their hosts." Berger says that he met with Namibian officials last summer to discuss the preliminary results, and also submitted them seventeen months ago for publication in the Namibian journal *Madoqua*.

John Robinson, vice-president of the Wildlife Conservation Society, based in New York, which helped to support Berger and Cunningham, claims that Namibia's actions represent "nothing but the muzzling of scientific enquiry". He says that the incident sets a dangerous precedent at a time when biologists are bringing science to bear on conservation policy, a field traditionally driven by little more than the whims of individuals. "This is clearly a case where science and conservation policy have got mixed up," says Robinson.

"A government can't say that it is taking a scientific approach to resource management if it takes sanctions against credible scientists whose research results conflict with government policy," says Peter Brussard, president of the US-based Society for Conservation Biology.

Officials from US Agency for International Development and the US embassy in Windhoek have raised the incident with Namibian environment minister Hanno Rumpf, and say he is adamant that the pair will not be authorized to continue their research. Berger and Cunningham now plan to finish writing up their Namibian work and move on to a new topic. "We want something that isn't so controversial," says Berger.

Colin Macilwain

US and China resume talks on cooperation

Washington. Chinese and US science delegations met in Washington last week for the first time since their previous biannual meetings were suspended in the wake of the 1989 Tian'anmen square massacre.

The meeting of the Joint Commission on Scientific and Technological Cooperation discussed cooperative research in the areas of environmental technology, energy efficiency, drug development and advanced materials. An agreement on joint research in marine and fisheries science and technology was renewed and an accord on clean coal research signed.

An official of the White House Office of Science and Technology Policy said that the four areas identified for future joint projects are "illustrative, not com-

prehensive", and are meant to alert government agencies to opportunities for future cooperation. "It's really up to the technical agencies and the private sector to pursue these opportunities as they see fit."

Twenty-seven protocols are already in place, which typically allow for cooperation between a single Chinese science agency and its US counterpart in a specific area of mutual interest. The meeting, co-chaired by John Gibbons, the US presidential science adviser, and the Chinese science minister, Song Jian, was one of a number of activities planned to lead up to the decision to be made in June by the US President, Bill Clinton, on whether to renew China's 'most-favoured nation' trading status.

Tony Reichhardt

Hand-telephones face US radiotelescope ban

Washington. The US Federal Communications Commission (FCC) has proposed a prohibition on the use of hand-held telephones which communicate via satellites within 100 miles of selected radiotelescopes — including large dishes in Puerto Rico, West Virginia and New Mexico — when certain observations are in progress. The new rules have been drawn up in response to the concerns of radioastronomers about the threat of 'noise pollution' from proposed satellite systems in low Earth orbit.

In addition to restrictions on telephone use near large facilities, the rules would create smaller protected zones around each of the ten antennas that make up the Very Long Baseline Array.

More than half-a-dozen companies, including Motorola, TRW and Loral, have announced plans to launch large constellations of small satellites to act as telephone relays in low orbit. The FCC has allocated two radio bands to these proposed systems: that from 1,610 to 1,626.5 MHz for 'uplink' to the satellites, and that from 2,483.5 to 2,500 MHz for transmissions down to the ground.

The uplink band includes one of the

spectral lines of the hydroxyl radical, which is of particular interest to astronomers because it holds clues to the nature of interstellar matter and star formation. To reduce radio noise, the National Science Foundation (NSF)'s electromagnetic spectrum management office would notify satellite operators of any planned observations at these wavelengths so that they could switch the uplink temporarily to another frequency or take some other action to avoid interference.

The downlink band is not used heavily for radio astronomy. But the spectral region around its second harmonic — near 5,000 MHz — is used for continuum observations. The FCC rule would limit the amount of noise satellites produce in this and other protected regions outside their main transmitting frequency.

Tomas Gergely, NSF programme manager for electromagnetic spectrum management, admits that the filters to block these out-of-band signals might cost "a fair amount of money". But he says that satellite owners have shown a willingness to accommodate the needs of astronomers.

John Windolph, a spokesman for the Motorola-led Iridium consortium, says that

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Arecibo already suffers 'noise' pollution.

"it's the responsibility of those entering the spectrum at a later date to co-ordinate with those who were there first". He believes that all the satellite systems will support the rule being proposed by the FCC.

The new rules are based on the outcome of negotiations that took place last year between representatives of the satellite communications industry and radioastronomers. The National Research Council's Committee on Radio Frequencies (CORF) is to file a letter in support of the rule, and expects no major opposition from the satellite owners. (The proposed rules are open for public comment until 5 May.)

Michael Davis, project scientist for upgrading the Arecibo Observatory in Puerto Rico and chairman of the CORF, describes the negotiations as a good model of the way in which astronomers can work with industry to find mutually satisfactory solutions. "You don't simply throw up your hands in horror and try to fight Wall Street," he said.

Arecibo has a petition before the FCC to establish a 'coordination zone' under which the observatory would automatically be notified whenever anyone in Puerto Rico applied for a broadcast licence. In the past, astronomers at Arecibo have had to deal with radio interference caused by everything from cellular telephones to a nearby religious television station.

US military satellites designed to detect nuclear bursts pose another problem, according to Davis. He estimates that five per cent of the observing time at Arecibo is lost because of signals from these spacecraft. Although satellite operators are meant to notify astronomers before such signals are transmitted, they do not always do so.

One piece of good news for radio observers is that managers of the Russian GLONASS navigation satellites, one of the worst offenders in the area of noise pollution, have agreed to shift its transmission frequencies. The decision was reached last autumn, after years of protest from the scientific community.

Tony Reichhardt

CERN seeks firm commitment to LHC

Munich. The council of the European Laboratory for Particle Physics (CERN) last week reaffirmed its intention of approving the building of its Large Hadron Collider (LHC) at its meeting in June. But the council failed to reach agreement on the precise wording of the resolution on which the members will vote. Some member states are keen that the wording should include a firm commitment to the construction of the LHC, regardless of whether additional funding is promised by non-member states.

Provisional plans are that the LHC would be built over the ten years 1995–2005. Under current estimates, it will cost 2.6 billion Swiss francs (US\$1.8 billion), which is SFr500 million more than CERN's 19 member states would pay out of their regular contributions during this period.

Christopher Llewellyn-Smith, CERN's director-general, is now trying to raise the shortfall from non-member states, including the United States, Japan and Canada.

If Llewellyn-Smith fails to raise the additional funds, his fall-back is to extend the construction phase by two years (see *Nature* 366, 714; 1993). But he is finding it difficult to persuade non-member countries to commit themselves to the project because it has not yet received approval from CERN members themselves.

Delegates at last week's council meeting approved the substance of a resolution pro-

posed by Llewellyn-Smith endorsing this strategy, but said that they needed a few more weeks to fine-tune its wording. In particular, the council members indicated that they want to avoid any ambiguity over the fact that their approval of the LHC will not depend on the promise of outside funding, insisting that such funding should be looked on as a 'bonus' which would make possible an optimal timetable.

The resolution asks for a comprehensive review of the LHC's proposed construction programme in 1997, when the first major outlay for the purchase of magnets is scheduled. By this time the extent of cooperation with non-member states should be clearer.

In particular the US is expected to have reached a firm decision by that date about its own high-energy physics programme, now that it has scrapped plans for the Superconducting Super Collider (SSC). This makes it possible for CERN to construct a definitive timetable for the remaining activities.

● There was celebration among LHC scientists last Thursday when LHC's first 10-metre prototype superconducting magnet, developed by CERN and the Italian Institute for Nuclear Physics, exceeded hopes by reaching a field of 8.67 tesla, 0.02 tesla higher than its target. The magnet, one of seven prototypes designed at CERN, reached 8.73 tesla in a second test, maintaining this level for 15 minutes.

Allison Abbott

Cancer institute to tighten control of trials

Washington. The US National Cancer Institute (NCI) plans to strengthen its monitoring of clinical trials following the recent public outcry over the discovery of fraudulent data in a trial evaluating the treatment of women with breast cancer.

Samuel Broder, director of the NCI, last week told the House of Representatives' subcommittee on oversight and investigation, chaired by John Dingell (Democrat, Michigan), that he accepted responsibility for the fact that the NCI had not been quicker in sorting out problems with the trial.

But he told Dingell that a reanalysis by the NCI of the original clinical trials indicated that the fraudulent data did not invalidate the conclusion of the trial. This showed that lumpectomy followed by radiation therapy is a preferable treatment for most women with early cancer to the more disfiguring and physically disabling mastectomy.

The lumpectomy trial is one of several exploring treatment for breast cancer, each involving many hospitals, that are being co-ordinated by the National Surgical Adjuvant Breast and Bowel Project (NSABP), set up 25 years ago at the University of Pittsburgh under the leadership of Bernard Fisher.

Several other large trials have reached the same conclusions as the NSABP. But women's health advocates told Dingell's committee that the previous lack of openness about the fraudulent data, which were submitted to the NSABP by Roger Poisson of St Luc's Hospital in Montreal, meant that many women are now worried about their decision to choose a lumpectomy.

Last year, the Department of Health and Human Services' Office of Research Integrity (ORI) concluded that Poisson altered the records of 15 per cent of his patients over 13 years to make them eligible for inclusion

in the trial. Concern about the NSABP's failure to spot this earlier was heightened at the end of last month when NCI officials found a discrepancy in data submitted for another of the project's clinical trials. These came from St Mary's Hospital, also in Montreal, and arose from a trial of the effectiveness of the drug tamoxifen in preventing breast cancer in women known to be at a high risk of developing the disease.

The ORI is now investigating the tamoxifen data, and the NCI has asked the University of Pittsburgh to replace Bernard Fisher — who some believe is being made a scapegoat for the whole affair — as the NSABP's principal investigator.

Broder stressed that Fisher was being replaced for administrative failures, not scientific misconduct. The NCI is annoyed that Fisher took nine months to inform them of problems with data in the lumpectomy trial, and failed to notify them when his staff found discrepancies in the tamoxifen trial.

It is also upset that he did not promptly submit for publication a reanalysis of the lumpectomy trial data excluding Poisson's contribution, and that the NSABP last year stopped checking samples of the data points it received against the originals held by hospitals, a standard technique used to monitor the quality of the data that hospitals send in to groups controlling large clinical trials.

As a result, the NCI told the NSABP at the end of March to suspend recruitment to all of its trials. NCI staff are now reviewing

all of the original data at the 400 participating sites of the lumpectomy trial.

Fisher, who is in his mid-seventies, was too ill to attend last week's hearing. But he released a statement saying that, if there had been any evidence that the conclusions of the NSABP studies were affected by the St Luc's data alterations, he would have reported it immediately. "In retrospect, this proved to be a mistake," said Fisher. "The lack of publication raised unnecessary fears among breast cancer patients."

Fisher has a number of supporters. Richard Peto, for example, professor of medical statistics and epidemiology at the University of Oxford, has sent a letter backing him to the *New England Journal of Medicine*. Peto says he is particularly concerned that more aggressive auditing of data will add heavy costs to large-scale randomized clinical trials, even though these are essential for uncovering effects that are small yet significant for treatment.

But in an attempt to allay public criticism arising from the Poisson case, Broder told the congressional subcommittee last week that the NCI would make more unannounced spot checks on hospitals participating in large trials. In particular, the institute will focus on the new tamoxifen trial, which is already highly contentious in both the United States and Britain. Tamoxifen is known to reduce the occurrence of new cancers in women already treated for breast cancer, and researchers hope that it may prevent cancer in women at high risk of contracting breast cancer. But the drug has resulted in death from cancer of the uterine lining in some patients.

The NSABP was informed of the first such death in 1991, but did not inform the NCI until August 1993. This information is only now being included in informed consent forms that women sign before a trial.

Under close questioning by Dingell, Broder accepted that the NSABP could have passed this information to the NCI in early 1992. He promised that the institute will improve the flow of data about drug toxicity between itself and bodies such as the NSABP that manage and analyse large clinical trials.

Researchers still face the question of whether the new data mean that tamoxifen should be withheld from healthy women at high risk of breast cancer, even though the benefit still outweighs the risk for women who have already had breast cancer.

Last week, the Food and Drug Administration required that tamoxifen be re-labelled with a warning that it poses a higher risk of cancer of the uterus than previously thought. Ed Sondik, acting deputy director of the NCI, says the institute will evaluate the information now available to decide whether the criteria for participating in the trial should be altered. **Helen Gavaghan**

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Broder: promising
better data flow

Bill Branson

BASF opens biotech laboratory in the US

Munich. The chemical company BASF last week formally opened its first major pharmaceutical research centre outside Germany, in Worcester, Massachusetts. The centre will carry out research in cancer and diseases of the immune system, and manufacture some biopharmaceutical products.

The decision to build the US\$100-million BASF Bioresearch Corporation (known as BBC) in the United States was partly a response to the difficulties that chemical companies have experienced in manufacturing bioengineered products in Germany. In 1988, BASF's plans to build a production plant for recombinant TNF α , a potential antitumour agent, were blocked because of the country's strict gene laws. The company decided to transfer biotechnology research across the Atlantic, but, not everything has

gone according to plan. Falling profits in BASF's pharmaceutical division have slowed down the development of the centre. It opened with 160 staff and will not reach its full capacity of 450 for several years.

Furthermore, the centre's original aim of developing and producing mostly protein-based therapeutics has been modified as a result of the disappointing clinical performance of many such compounds. As a result, the centre's biotechnology production facilities, currently being used only to manufacture BASF's new anticoagulant PEG-hirudin, are underutilized.

BBC is seeking partners to rent out excess capacity to other companies. But its president, Robert Kamen, says he is not disappointed by the scaled down plans, which he describes as "realistic". **Alison Abbott**

Haemophiliacs seek charges in AIDS scandal

Tokyo. The scandal in Japan about the supply of HIV-infected blood products to haemophiliacs in the 1980s has taken a dramatic turn with a request from a group of 217 people, made up of haemophiliacs and their relatives, for the Tokyo District Prosecutor's Office to charge a leading haemophilia specialist with "wilful negligence".

The action, which is based on the infection of two haemophiliacs with HIV, is the first attempt in Japan to institute criminal proceedings against those who were involved in the country's blood product policy during the early to mid-1980s, when nearly 2,000 haemophiliacs were infected with HIV through the use of contaminated blood coagulants.

In a legal document called a *kokuhatsumo*, the group asks for charges to be made against Takeshi Abe, vice-president of Teikyo University in Tokyo, who in 1983 headed a group set up by the Ministry of Health and Welfare to help formulate policy on blood products (see *Nature* 367, 584; 1994).

Two civil court cases, in which almost a hundred haemophiliacs suffering from AIDS are suing the government and pharmaceutical companies, have been under way for several years in Osaka and Tokyo district courts (see *Nature* 364, 181; 1993). But this is the first attempt to launch criminal charges against an individual.

The group claims that although Abe knew by late 1984 that a large percentage of his patients were infected with HIV, he continued to treat uninfected patients with blood products that had not been treated with heat to kill the virus. As a result, two further patients were infected between late

1984 and the middle of 1985.

The group also argues that Abe was well aware by late 1984 of the potential dangers of non-heat-treated products. As a result, they say, he showed wilful negligence (*mihitsu no koi*) in continuing to prescribe these products to patients.

The group's case hinges on a paper that Abe published with six colleagues in the *Journal of the Japan Society of Blood Transfusion* in 1988 (34, p.332-339). The paper traces the appearance of antibodies to HIV in the blood samples of patients treated in Abe's department.

Among them are two cases that the paper claims showed negative results in early to mid-1985 with a variety of tests, but later became positive in all tests. The first individual was negative in tests on a blood sample collected early in June 1985, but positive in some tests for a sample collected six and a half weeks later. The second was negative in February 1985, but positive in most tests three and a half months later.

Arguing that antibodies to HIV usually appear within 8 weeks of infection, the group concludes that these two patients must have been infected sometime between early December 1984 and mid-1985.

But in August 1984, Abe sent blood samples of 48 patients to Robert Gallo of the National Cancer Institute in the United States who, together with Luc Montagnier of the Pasteur Institute in France, had discovered the virus responsible for AIDS earlier that year. The group claims that the results showed that 23 of Abe's patients were infected with HIV. According to testimony in the Tokyo District Court by Mutsuyoshi

Kazama, a colleague of Abe's at Teikyo University and a co-author of the 1988 paper, these results were known to Abe by at least 8 November 1984, the date of the Fourth International Symposium on Haemophilia Treatment in Tokyo.

As a result of Gallo's results, the group argues that Abe must have known of the possibilities of HIV infection from non-heat-treated blood coagulants by that time. Indeed, the group argues that Abe should have been aware that HIV might be present in blood coagulants well before this, as he already had a haemophiliac patient with typical AIDS symptoms in 1983 (*Nature* 367, 584; 1994).

On the advice of the Tokyo District Prosecutor's office, Abe is refusing to comment on the haemophiliac group's charges. But there are a number of weaknesses in the charges. First, the identity of patients in the 1988 paper is unknown, and it is therefore not known whether the two patients referred to in the charges are alive or dead — or indeed whether they have contracted AIDS.

Second, although it is generally accepted that HIV antibodies almost invariably appear within 8 weeks of infection, there have been reports of antibodies not appearing until 3 to 6 months after infection. This would push back the date of earliest possible infection for the two cases referred to in Abe's 1988 paper, making it less certain whether infection occurred after Abe received Gallo's test results, at least in the second (earlier) case of infection.

Abe himself has long avoided commenting publicly on Japan's blood scandal. On the rare occasions that he has talked to the media, his answers have been unclear and evasive, making him an easy scapegoat for the tragedy in the Japanese press. His colleagues say he shows great care and compassion for his patients, and reject the charges against him.

But Toshihiro Suzuki, a lawyer for the haemophiliac group, says that its aim is not to put the 77-year-old Abe in jail. Rather, it wants to force an official government enquiry into the whole tragedy, which led to nearly half of Japan's 4,000 haemophiliacs being infected with HIV.

The group has already appealed to the Minister of Health and Welfare to set up an investigation, but so far without response. It has therefore decided to press for criminal charges against Abe, who is at the centre of the whole affair.

But a quick decision from the prosecutor's office seems unlikely. Suzuki says that only one prosecutor has been assigned to the case as the office is currently investigating numerous money scandals involving leading politicians. Furthermore, this one prosecutor has many other cases to cover.

David Swinbanks

Physicists' group split on Stasi case

Washington. A US physicists' human rights group is likely to drop its investigation into the case of a former agent of the East German secret police, the Stasi, who lost his university job, after being told by the German Physical Society in Cologne that it considers his rights have not been violated.

The Committee on the International Freedom of Scientists of the American Physical Society (APS) has been looking into the case of Dietrich Demus, who lost his physical chemistry chair and professor's position at Halle University in 1991 when Stasi files showed that he had worked as an informer.

Demus now works in Japan. He is one of a number of scientists in the former East Germany who say that they have been unfairly treated by a process which, under the 1989 German unification treaty, allows them to be dismissed for

having worked with the Stasi (see *Nature* 359, 762; 1992).

But at a session of the annual meeting of the APS in Arlington, Virginia, this week, some members of the society criticized the human rights committee for devoting scarce resources to Demus's case. Herman Winick, who has led a subcommittee dealing with the case, said that the committee needed to consider all cases on their merit, and had not yet intervened on Demus's behalf.

Outgoing committee chairman Joe Birman of the City College of New York told the session that the human rights agenda facing it worldwide had not diminished, as might have been expected with the collapse of communism in eastern Europe. The new chair of the committee is the formerly imprisoned Chinese dissident physicist, Fang Lizhi.

Colin Macilwain

Shake-up urged for UK public research labs

London. Sweeping changes in the organization of Britain's government-run research institutions have been proposed by a team of civil servants that has for the past three months been carrying out an 'efficiency scrutiny' of the way in which such institutions are run.

In particular, the scrutiny team has proposed that separate institutes sharing a common research interest — for example food production or the marine environment — be brought together as agencies with a unified management structure.

Specific proposals cover most of the institutes run by the National Environment Research Council (NERC) and the newly formed Biotechnology and Biological Sciences Research Council (BBSRC). Although the agencies would still be 'owned' by the respective research councils, the new arrangements would sharpen the distinction between the 'sponsor/customer' role of the councils, and the 'supplier/contractor' role of the institutes.

In each case, a chief executive would be appointed over and above the current institute directors, with a specific remit to streamline organization and ensure "continuing economy, efficiency and effectiveness", including "any necessary refocusing

of the missions of the laboratories".

No proposals are made for the Rutherford Appleton and Daresbury Laboratories run by the Engineering and Physical Sciences Research Council, which are the subject of a separate review. But members of the review team say there would be an "advantage" in applying a similar approach to institutes run by the Medical Research Council (MRC) "to reflect the same sponsor/supplier division".

The preliminary conclusions of the review team, which were presented to government scientists and research council officials last Friday, do not propose the widescale privatization that many had feared.

Indeed, the group says that its scrutiny of 53 government laboratories has found "no clear-cut cases of suitability for early privatization" over and above those already identified in departmental reviews (such as the Transport Research Laboratory).

But, picking up on a suggestion first made last summer in a report by Sir Peter Levene, the government's efficiency adviser, and Bill Stewart, its chief scientific adviser, the group endorses the idea that the institutions be combined into a number of separate groupings managed relatively

independently of their sponsoring bodies.

The main research institutions supported by the NERC, for example, would be grouped as a single agency coordinating the activities of all government research institutions concerned with the non-marine environment, ranging from the British Geological Survey to the laboratories currently run by the Forestry Commission.

Institutions dealing with the marine environment would form a separate agency 'owned' by the Scottish Office (SO). This would include not only bodies currently run by the SO, such as the Fisheries Research Service, but also two NERC institutes, the Dunstaffnage Marine Laboratory and the Plymouth Marine Laboratory.

The BBSRC would 'own' government laboratories focusing on biotechnology and the biological sciences. Complementary to this would be a separate grouping of food and agriculture laboratories, primarily those now run by the Ministry of Agriculture Food and Fisheries, but also possibly including the Natural Resources Institute of the Overseas Development Agency. There would also be a grouping 'owned' by the Home Office of laboratories concentrating on forensic science.

In proposing these initial five agencies — and suggesting that a further two might be created for medical research and health services research — the review group has backed away from the alternative proposal made in the Levene/Stewart report creating a single Civil Defence Agency.

But it has suggested a number of government laboratories that might be taken over by neighbouring universities, including the Institute of Virology and Environmental Biology near Oxford and possibly the BBSRC's Institute for Animal Health at Babraham, close to Cambridge University.

Finally, the review group suggests that there should be "some scope for amalgamation" between the Public Health Laboratory Service and the laboratories run by the National Health Service.

In general, the scrutiny team's conclusions have been welcomed with a certain relief from those who had feared that it might lead to a full-scale sell-off of government laboratories. But there remains concern both about the implications of introducing a new level of bureaucracy, and about the changes that may be imposed on institutions under the new arrangements in order to prepare them for later privatization.

In particular, labour unions representing scientific staff at the institutes are nervous that a considerable number of jobs could be lost through the application of the scrutiny team's various proposals for achieving what it describes as "savings/flexible use of manpower" by linking together laboratories within a single agency. **David Dickson**

Jobs to go as the private sector moves in

London. Michael Heseltine, the president of Britain's Board of Trade — and a potential challenger to the prime minister, John Major — announced last week that he had decided to take steps to privatize three of the United Kingdom's main government research laboratories now owned by the Department of Trade and Industry (DTI).

The National Physical Laboratory (NPL), the government's main body for developing and disseminating national standards and based at Teddington outside London, will remain under the DTI's ownership for the time being, but its management will be contracted out to the private sector.

The Laboratory of the Government Chemist, which carries out functions from drug detection for the customs service to assisting the police force with DNA analysis and shares the same site as the NPL, will be set up as an "independent non-profit distributing company" — although it could be sold outright "if a suitable buyer comes along".

The National Engineering Laboratory in Glasgow, which has already been slimmed down in preparation for eventual privatization, will be put on the market in the summer of next year.

Heseltine's announcement had been widely anticipated. The government argues that privatization is needed to ensure that the laboratories become "more focused in their work, more sensitive to their custom-

ers' requirements, and more cost-efficient".

Indeed, despite concerns in both the scientific and industrial communities (see *Nature* **363**, 196; 1994), Heseltine is widely believed to have argued strongly for immediate privatization, and only backed off reluctantly when the desirability of such a step was challenged in a report prepared for the DTI by management consultants KPMG.

Nevertheless, the government's decision has generated a strong reaction from the opposition Labour party. Michael Meacher, the shadow minister for science, condemned the move as an "ideologically driven course" that will "undermine the industrial regeneration the country needs so desperately".

Cuts in DTI support are likely to lead to considerable job losses among scientists working at the three laboratories. The Institute of Professionals, Managers and Specialists, which represents the 700 scientists at the laboratories, describes the privatization decision as a "wanton destruction of a national resource", and says it runs "counter to the laboratories' mission to provide independent advice on technical issues".

Heseltine, however, remains adamant that the most important task facing each of the laboratories is to raise their ability to respond more flexibly to the needs of British industry, and that this can be best done from the private sector. □

Science versus anti-science

SIR — John Maddox's article about anti-science (*Nature* 368, 185; 1994) raises a fundamental issue: how to counter the increasingly vocal fringe of society pumping energy into the preconceptions of the majority.

I have no formula to offer, and can only suggest that a closer definition of the focus of majority fears might help science to find an antidote to stop the rot while efforts are mounted to bridge the abyss of ignorance.

It appears to me as a moderately informed observer — for 35 years from within the chemical industry — that the long-standing equilibrium between "religion and astrology etc." on the one hand and rational objectivity on the other became unstable at about the time that science began to express serious concern about the impact of humanity on the life support systems of the planet.

This equilibrium between irrational subjectivity and rationality had, since time immemorial, been mediated essentially by intellectuals of one persuasion or another. Clearly it has shifted since the days of Thomas Aquinas, but by the end of the nineteenth century it was still the basic condition in which philosophical discourse took place. Religion had conceded a great deal of ground, not least to Copernicus, but was still recognizable as a major force in evolute societies; elsewhere, it remained the principal controlling factor of human emotion and of enquiry.

The last decades of the twentieth century brought us two entirely novel influences that have upset the equilibrium: worldwide communications and the awareness of humanity as a potential danger to itself. Science alone conjured these factors out of its deepest thinking, and in so doing has assumed responsibility for many of the longer-term consequences. Furthermore, the characterization, as it were by definition, of many aspects of human behaviour in evolute societies as contrary to Darwinian principles has led to defensive postures more strident than those related simply to arguments about population growth and survival of the fittest.

It should not surprise us, then, that the multitudes dislike the message, nor that there are those who exploit the communication miracle to broadcast fears and fantasies and emotive prescriptions for peace, prosperity and fulfilment. The prescription is: "Kill the messenger!"

To make matters worse, science is seen to have arrived with dirty boots, so it may well take more than a polemicist to avoid its lynching. Nothing less than a convincing assurance that it can "Save the Planet for Life" will suffice. Taking its cue from politics, science must make upbeat prom-

ises, preferably ones it can deliver in the short term, in a comprehensible language. Then, if the rot can be stopped, science must concentrate on building bridges to help mankind step across the abyss.

D. A. A. Fagandini
6 Alleyn Park,
London SE21 8AE, UK

SIR — Maddox pleads passionately for a more polemical defence of science — against anti-science. While sympathizing with his concern, I wonder if his proposed strategy would serve the intended purpose. Will science benefit from lowering itself onto the battlefield of its presumed enemies? Let's face it: astrology is at least as old as science, whatever definition you choose for that noble endeavour. Science has flowered all along, with ups and down, in spite of the presence of the irrational. I refuse to believe that religion and superstition compete with science in the same market for whatever livelihoods one might consider. What I regard as true science has always distinguished itself from such sectarian pursuits by its openness to challenge, its lack of fanaticism — and its disunity (see J. Dupré, *The Disorder of Things*, Harvard University Press, 1993).

Irrespective of these issues of dignity, the present crisis of science is not due to recrudescence of anti-science. If anything, it's the other way round. The flattering public support of the scientific enterprise has its roots in the prevailing (relative) peace. War has always been — directly or indirectly — the single most important stimulant for science, materially but also by spurring inventiveness. Enlightenment may be what scientists cherish most by way of reward for their labour, but people and governments tend to favour more prosaic motives. As a scientist and a pacifist I thus find myself in a severe dilemma. Maybe what is needed now for scientists to save their cause is a lesson in humility.

Werner Sieber
rue du Botzet 3,
1700 Fribourg, Switzerland

SIR — Maddox writes that: "As the long (and soon to end) correspondence in *Nature* has shown, many professional scientists are deeply religious, often justifying their belief on the grounds that 'science cannot know everything.'" Later in the same paragraph, he writes, "... it may not be long before the practice of religion must be regarded as anti-science".

Where does Maddox propose to draw the line? If *Nature* views religious scientists as anti-scientific, will this make all their scientific work suspect and therefore unworthy of publication?

Elsewhere in the same issue is a review

by David Knight of two books about Robert Boyle (368, 200; 1994) which describes Boyle's well-known Christian faith. Does this make the famous chemist anti-scientific in the eyes of *Nature*? Many papers in the same issue use nomenclature devised by Linnaeus, another devout Christian. Finally, the sales office of *Nature* printed in the same issue as Maddox's remarks (Classified page 32) an announcement of deadlines altered "Due to the Easter recess. . .".

If *Nature* plans to draw the line in religion, then those of us who faithfully subscribe and occasionally contribute to its pages, but who also happen to be religious, need to know precisely where the line is to be drawn.

Forrest M. Mims III
433 Twin Oak Road
Seguin, Texas 78155, USA

SIR — You serve warning that to defend against anti-science you would end the "disgraceful . . . benign tolerance" afforded not only to astrology, but also to "exaggerated arguments" over nuclear power and genetics. Most strikingly, you observe that "it may not be long before the practice of religion must be regarded as anti-science".

How long, Sir? Some of us will need to know when to submit our manuscripts elsewhere, lest our practice of religion indicates to you that we regularly perpetrate "a pack of lies" (like astrology) or "false statements" (like those exaggerators). Shall your Guide to Authors soon require that we disclose our religious affiliations?

You say that the long correspondence on religion in *Nature* is "soon to end". How sad that your last word is a polemic attack on the religious among your readers and authors.

Peter Vibert
Rosenstiel Basic Medical
Sciences Research Center,
Brandeis University,
Waltham, Massachusetts 02254, USA

SIR — Mario Vaneechoutte (*Nature* 365, 290; 1993) refers to religion as a meme. The adjective "memetic" and the noun "meme" do not appear in the *Oxford English Dictionary*. There is little doubt that Richard Dawkins is the originator of the noun in 1976 in his famous book *The Selfish Gene*. This work is reprinted and is easy to find (1989). The definition of "meme" is on page 192. He defines it as a non-genetic replicator that flourishes only in the environment provided by complex brains. Personally I think Vaneechoutte is correct in his analysis of a complex question.

Frank W. Cousins
43 Emanuel House,
18 Rochester Row,
London SW1P 1BS, UK

Mystery of the poisoned expedition

John W. Earl and Barry V. McCleary

The Burke and Wills expedition through the interior of Australia in the nineteenth century ended in calamity. But the cause of death was more pernicious than anyone at the time had imagined: beriberi due to thiaminase poisoning.

In 1860–61, the explorers Robert O'Hara Burke, William John Wills, John King and Charles Gray became the first Europeans to cross the Australian continent from Melbourne in the south to the Gulf of Carpentaria in the north. But all except King died on the return journey. The report of the Royal Commission of 1861–63 that "endeavoured to ascertain the true causes of this lamentable result" concluded that the "deplorable sufferings and untimely deaths" might have been averted had not the whole expedition been so badly organized, with insufficient and unreliable back-up.

But what about the exact causes of death? It is now clear that the explorers suffered from beriberi, a disease caused by dietary deficiency of thiamine (vitamin B₁). They developed the disease while consuming foods containing large amounts of thiaminase I, an enzyme that breaks down thiamine. Wills's diary, recovered from their final camp at Cooper's Creek, contains a textbook account of the disease — probably the first and only complete description of thiaminase poisoning in humans.

Burke, the leader of the party, was a police inspector. His deputy, Wills, was a scientist who studied chemistry in 1852 with Dr John Stenhouse, a lecturer at St Bartholomews Hospital Medical School in London, England. Wills assisted Stenhouse with his work on food analysis and, after emigrating to Australia, was appointed to the Magnetic Observatory in Melbourne (1857–59) where he studied astronomy and meteorology¹.

The Burke and Wills expedition was set up by the botanist Ferdinand Mueller and other fellows of the Royal Society of Victoria "for transversing the unknown interior of the Australian continent". Its aims were to search for the lost expedition of scientist-explorer Ludwig Leichhardt, collect botanical specimens, identify new species of plants and animals and gather astronomical, meteorological and geographical data, to name but a few. The exploration committee prepared specific instructions for its team of specialists: "The Committee has caused the inclosed set of instructions to be drawn up, having relation to each department of science, and you [Burke] are requested to hand to each of the gentlemen a copy of the part more particularly relating to his department"¹.

On arriving at Menindie, the expedition

was, according to the Commission, "most injudiciously divided at that point by Mr. Burke", and at Cooper's Creek he split the party again, leaving behind all the scientists except Wills. Scientific aims were also abandoned as the expedition became a race to cross the continent



Wills — diarist of the explorers' disease.

before the south Australian expedition led by John McDouall Stuart¹. As the Commission pointed out:

Mr. Burke evinced a far greater amount of zeal than prudence in finally departing from Cooper's Creek before the depôt party had arrived from Menindie, and without having secured communication with the settled districts as he had been instructed to do; and in undertaking so extended a journey with an insufficient supply of provisions, Mr. Burke was forced into the necessity of overtaxing the powers of his party, whose continuous and unremitting exertions resulted in the destruction of his animals, and the prostration of himself and his companions from fatigue and severe privation".

With William Brahe left in charge of the relief party, only Burke, Wills, King and Gray set out from Cooper's Creek and successfully completed the journey to the north coast of Australia. Delayed by monsoons on their return journey and finding their rations depleted, the explorers began to live off the land. Freshwater mussels, abundant in the inland creeks, were gathered from the mud and roasted by Aborigines². As Wills observed: "The well worn paths, the recent tracks of natives, and the heaps of shells, on the contents of which the latter had feasted showed at once that this creek must be

connected with some creek of considerable importance". The explorers also consumed these mussels, although it is uncertain whether they roasted them or ate them raw: "proceeded up the creek; obtained some mussels near where [the camel] Landa died, and halted for breakfast"¹. The freshwater mussel *Velesunio ambiguus* contains a thiaminase I enzyme that breaks down thiamine³.

On returning to Cooper's Creek the explorers found that the relief party had deserted them. With supplies low, they began to eat nardoo, a flour prepared from the sporocarps of the nardoo fern *Marsilea Drummondii* by the local Aboriginal tribes¹. The clover-like fronds of the nardoo fern contain a hundred-fold more thiaminase than bracken-fern fronds, and the sporocarps of nardoo have two to three times more thiaminase activity than bracken-fern fronds³. Bracken poisoning due to consumption of bracken fern causes the staggers in horses⁴, and sheep that feed on nardoo in western New South Wales develop a similar thiamine-deficiency disease³.

Wills meticulously observed and recorded the explorers' symptoms and suffering in his journal. Gray, a British sailor, was the first to succumb on the return journey: "Halted 15 minutes for Gray who pretended he could not walk". Gray instinctively seemed to know he needed more of the precious flour ration, the party's main source of thiamine. "I found Gray behind a tree eating Skilligoollee. He explained that he was suffering from dysentery and had taken the flour without leave". Wills thought Gray was malingering but later notes: "This morning about sunrise Gray died. He had not spoken a word distinctly since his first attack"⁵.

Even before they began to eat nardoo, the other three explorers were showing preliminary signs of beriberi:

Our legs almost paralysed so that each of us found it a most trying task only to walk a few yards. Such a leg bound feeling I never before experienced and hope I never shall again. The exertion required to get up a slight piece of rising ground, even without any load, induces an indescribable sensation of pain and helplessness, and the general lassitude makes one unfit for anything. Poor Gray must have suffered very much, many times when we thought him shamming. It is most fortunate for us that these symptoms which so early affected him, did not come on us until we were reduced to an

exclusively animal diet of such an inferior description as that offered by the flesh of a worn out and exhausted horse [ref. 5].

The explorers recovered remarkably on the rations left by their relief party. But once they began collecting nardoo and preparing their own nardoo flour, their health progressively deteriorated. From weakness and pain in the legs, they developed metabolic insufficiency, muscle wasting and hypothermia until they were completely unable to move. Burke and Wills eventually died:

King out collecting nardoo. Mr. Burke and I at home, pounding and cleaning. I still feel myself, if anything, weaker in the legs, although the nardoo appears to be more thoroughly digested. . . found myself altogether too weak and exhausted; in fact, had extreme difficulty in getting across the numerous little gullies, and was at last obliged to camp from sheer fatigue. . . . The cold plays the deuce with us from the small amount of clothing we have. . . . Mr. Burke suffers greatly from the cold and is getting extremely weak. . . . My pulse are at forty-eight, and very weak, and my legs and arms are nearly skin and bone. I can only look out, like Mr. Micawber 'for something to turn up', but starvation on nardoo is by no means very unpleasant, but for the weakness one feels, and the utter inability to move oneself, for as far as appetite is concerned, it gives me the greatest satisfaction. Certainly, fat and sugar would be more to one's taste, in fact, those seem to me to be the great standby for one in this extraordinary continent, not that I mean to depreciate the farinaceous food, but the want of sugar and fat in all substances obtainable here is so great that they become almost valueless to us as articles of food, without the addition of something else [ref. 5].

In his last letter to his father, Wills writes that their food does not nourish them: "These are probably the last lines you will ever get from me. We are on the point of starvation, not so much from absolute want of food, but from the want of nutriment in what we can get". Wills was left alone to die in his humpy with a supply of nardoo¹.

King, the only member of the party to survive, also described the effects of thiaminase poisoning in his report to the Commission:

We gathered some nardoo and boiled the seeds as we were unable to pound them. . . . I had now to gather and pound for all three of us. I continued to do this for a few days but finding my strength rapidly failing, my legs being very weak and painful, I was unable to go out for several days. . . . From the time we halted Mr. Burke seemed to be getting worse, although he ate his supper. That night he spoke very little, and the following morning I found him speechless, or nearly so, and about eight o'clock he expired.

King was cared for by local Aborigines, living on nardoo, fish and game such as

crows⁵, until he was eventually rescued. From the description by Wills's father, Dr William Wills, it is clear that even after King had returned to Melbourne and regained his strength on a normal diet, he remained crippled. "On reaching Government House, King was assisted upstairs, for although he looked very healthy and robust, he was scarcely able to stand"¹. King had developed a permanent peripheral neuropathy as a result of prolonged thiamine deficiency.

Burke and Wills prepared nardoo in the traditional European way for grains by grinding and cooking. But the nardoo fern is well adapted to the extreme temperatures of inland Australia and its thiaminase enzyme is very resistant to heat³; nardoo spores will still germinate from sporangia that have been boiled in water for 15 minutes⁶.

By contrast, Aborigines of the Cooper's Creek area prepared nardoo by grinding it to a thin paste with water and keeping it out of contact with other organic material, a custom described by B. Kerwin: "Then there is the nardoo; they crush it and then rock it in a coolamon. Then they pour water on it and eat it with the water. What then? Well, they eat it by spooning it into their mouths with a mussel (shell), not with a coolibah leaf or with bark, only with a mussel (shell)"⁷. The Aborigines drank the water used in the preparation and apparently used the raw flour to make cakes and bread. "On our arrival at the camp, they led us to a spot to camp on and soon afterwards brought us a lot of fish and bread, which they call nardoo". The flour was also stored for later consumption. "I found some gunyahs where the natives had deposited a bag of nardoo sufficient to last me a fortnight"⁵.

The thiaminase I of nardoo requires endogenous co-substrates such as proline, hydroxyproline or adenine; scarcity of these substances in nardoo severely restricts the enzyme's action³. The kinetics of nardoo thiaminase³ shows that dilution with water rapidly diminishes the enzyme's activity: enzyme, thiamine and co-substrates are all diluted out. The Aboriginal method of preparing nardoo — grinding with water and preventing contamination by extraneous sources of co-substrates — would have reduced the enzyme's action. (One of us (J. W. E.) tried to get hold of samples of nardoo from Cooper's Creek but was prevented by floods. Plants have now been obtained from a nursery in Sydney, and as soon as they produce sporocarps it should be possible to compare the amounts of thiamine in different preparations).

Unfortunately, Wills failed to appreciate the need for leaching of nardoo flour with water, even though the technique had been demonstrated to him by an Aborigine he referred to as Pitchery:

The fish being disposed of, next came a supply of nardoo cake and water until I was so full as to be unable to eat any more, when Pitchery, allowing me a short time to recover myself, fetched a large bowl of the raw nardoo flour mixed to a thin paste, a most insinuating article, and one that they appear to esteem a great delicacy [ref. 5].

Wills was nevertheless aware of the need for essential nutrients. He identified the portulac plant *Portulacae oleraceae* and convinced the other explorers to eat its leaves to prevent scurvy or "Barcoo rot".

Half a century after the Burke and Wills expedition, a substance termed "vitamine", which could cure the weakness of beriberi, was isolated from rice, bran, yeast and other foods⁸. Thiamine occurs in the outer husks of most cereal grains and its discovery led to rapid progress in isolating and identifying other vitamins. But in 1861, stranded at the edge of the desert in central Australia, Wills, carefully observing his situation, glimpsed and recorded in his diary a fundamental truth:

We were not long in getting out the grub that Brahe had left, and we made a good supper off some oatmeal porridge and sugar. This, together with the excitement of finding ourselves in such a peculiar and almost unexpected position, had a wonderful effect in removing the stiffness from our legs. Whether it is possible that the vegetables can so have affected us, I do not know, but both Mr. Burke and I remarked a most decided relief and a strength in the legs greater than we had for several days. I am inclined to think that but for the abundance of portulac that we obtained on the journey, we should scarcely have returned to Cooper's Creek at all [ref. 5]. □

John W. Earl is in the Department of Biochemistry, Royal Alexandra Hospital for Children, Pyrmont Bridge Road, Camperdown, Sydney, New South Wales 2050, and Barry McCleary, formerly with the New South Wales Department of Agriculture, is at Megazyme (Aust) Pty Ltd, 2/11 Ponderosa Parade, Warriewood, New South Wales 2102, Australia.

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Triplet repeat genes raise questions

The novel mechanism for genetic mutation in Huntington's disease offers no comfort for neo-Lamarckians, but raises many other questions likely to prove difficult as well as intriguing.

IS THE Human Genome Project about to rehabilitate Lamarckian inheritance, or at least the inheritance of some acquired characteristics? That seems to be the unspoken question attending the recognition in the past few years of the role of repetitive and simple DNA sequences in the inheritance of several diseases, all of them (so far) conditions with neurological consequences.

Seven conditions have so far been well characterized, including fragile X syndrome, Huntington's disease (HD) and myotonic dystrophy, in which the underlying genetic mutation is an increase of the number of nucleotide triplets outside its normal range. In HD, for example, the gene for a protein now called huntingtin (for want of a clear idea of its function) on the shorter arm of chromosome 4 normally contains between 6 and 37 repeats of the nucleotide sequence CAG (which codes for the amino acid glutamine), but people who inherit from either parent a chromosome 4 in which the number of CAG repeats lies above that range are at risk of developing HD.

Indeed, in HD and the other diseases, there is a close inverse correlation between the number of CAG repeats and the age of onset. With 40 repeating units, the onset of HD is likely to be delayed until middle age, but with a chromosome containing more than 50 repeats, HD is likely to appear in a person's teens or even infancy. HD is dominantly inherited; either a maternal or a paternal chromosome 4 with an excess of repetitive CAG units is likely to be damaging.

Worse than that, the phenomenon of what the geneticists call anticipation applies. In other words, the age of onset tends to be reduced and the severity of the disease increased in successive generations in families susceptible to the disease. That in turn implies that the longer the repetitive units in one generation, the longer still they are likely to be by the next.

Is that not simply Lamarckian? The old chestnut about the length of the giraffe's neck almost literally applies. Lamarckians would have it that earlier generations of animals stretched their necks to reach into trees and were then able to pass on this advantage to their offspring; Darwinians, while readily confessing that there is no evidence to show what would have been the selective advantage of longer necks at intermediate stages, would insist that the eventual inheritance of this characteristic would have arisen from the natural selection of naturally arising variations.

Sadly (at least for Lamarckians) the analogy is false. Both Darwinians and Lamarckians are concerned with the evolution of physical characteristics, aspects of the phenotype. Precisely what features of the genome are responsible for them is strictly speaking irrelevant to last century's argument, although it may have a bearing on the more recent question whether the concept of the 'selfish gene' is a faithful representation of the darwinian process. For do not CAG repeats that tend to lengthen *in situ* have an intrinsic selfishness? Again, the analogy is false; for a selfish gene to succeed, it must populate as many organisms as possible, not debilitate a few.

In reality, of course, HD is a familial disease and the susceptibility is inherited by the mendelian rules that apply to genetically dominant conditions. Moreover, it must be plain that neither the particular CAG repeat involved in HD nor triplet repeats in general can be inherently unstable, for then we should have to face the chilling prospect that all families would eventually carry HD, as well as, no doubt, fragile X syndrome and the other conditions. From there it would be a short step to the conclusion that *Homo sapiens* will soon come to a sticky end by the prevalence of neurodegenerative disorders whose potential is embodied in its genome.

Two questions then arise. Given that the length of the repetitive CAG sequence in the HD gene is an unambiguous determinant of disease, what is the physiological connection between the length of the repeat and the damage done in the brain (chiefly the death of neurons)? And what feature of the genome is responsible for the instability of the CAG repeating unit in HD families, or is the true genetic cause of familial HD?

What follows is a necessarily random gleaning from the recent literature that seems to throw some light on that question. Remarkably, as it happens, the nucleotide sequence of huntingtin as originally published (*Cell* 72, 971–983; 1993) showed that immediately downstream of the CAG repeat is a different trinucleotide repeat, formed of the nucleotides CCG (coding for the amino acid proline). And now it turns out that the length of this repetitive unit is variable between individuals ('polymorphic' as they say in the trade); it may even be part of the explanation why the CAG repeating unit is unstable in HD families.

Two separate reports in the same issue of *Human Molecular Genetics* seem to show, when taken together, that the polymorphism of the CCG repeat helps to determine the stability of the CAG repeat. Thus Susan A. Andrew *et al.* (*Hum. molec. Genet.* 3, 65–67; 1994) from the University of British Columbia show that in 205 normal people, the CCG repeating unit can contain 7, 9, 10, 11 or 12 repeating units, of which 7 and 10 respectively account for 67 per cent and 30 per cent of the population, but that HD patients almost invariably have 7 CCG units.

At the same time, Lili H. Barron *et al.* from the Human Genetics Unit of the University of Edinburgh describe a study of the CCG polymorphism in HD patients and normal people from Scotland (*Hum. molec. Genet.* 3, 173–175; 1994) in which there appear to be five different versions of the CCG repeating unit. The numbers are not exactly comparable with those in the Vancouver study, because the Edinburgh group has measured both the first stretch of CCG repeats and a second separated from it by 18 nucleotides, but again they find that HD patients invariably carry just one of these, that which happens to be most common in the Scottish population.

One should not jump too quickly to conclusions, and it cannot be the case that the most common CCG repeating unit is the sole decisive determinant of the instability of the CAG repeat that lies upstream of it, for 60 per cent of us would then be at risk of HD. But the 7-CCG repeating unit (in the Vancouver nomenclature) looks as if it is a necessary, but not a sufficient, condition for instability. That should be an entertaining conundrum for structural biologists, but the search for the other determinants of instability remains a problem for geneticists.

On the first question, the mechanism by which the CAG repeat causes HD if there are more than 40 units in it, there is less to say. Because HD is dominantly inherited, there must be 'gain of function', but that says nothing of what the function may be. So it is as well that people have made a start on telling where huntingtin is to be found in the body. A group from Leiden and Rotterdam (A. T. Hoogeveen, *et al. Hum. molec. Genet.* 2, 2069–2073; 1993) has, for example, used antibodies against a huntingtin peptide to show the presence of huntingtin in brain, testis and other tissues. The distribution appears the same in normal people and those with HD. The protein appears in the nuclei of neurons but not of other somatic cells.

That, sadly, is only a beginning. And no amount of elegant histochemistry will tell what is the natural function of huntingtin. What gain of function there can be that enables the aberrant protein to kill neurons is something else again.

John Maddox

Diversity begets productivity

Peter Kareiva

THE 'equipment' of ecology often amounts to little more than a butterfly net and a field notebook. But with the publication of an experiment by Naeem and colleagues on page 734 of this issue¹ — the first major research results from the Ecotron facility at Silwood Park, Berkshire, UK — ecological science enters the big league, the world of particle accelerators and orbiting telescopes.

The Ecotron consists of 16 environmental chambers, and Naeem *et al.* have used this unique system to replicate terrestrial communities that differ only in their biodiversity. They find that the more diverse communities exhibit higher rates of primary production and respiration, and thereby provide the first unambiguous documentation of the effects of biodiversity on ecosystem processes. Although ecologists have long speculated that biodiversity provides services that are crucial to energy flow and life-sustaining biogeochemical processes, experiments addressing this issue have been hindered by lack of control over the communities being contrasted and by poorly replicated experimental units.

Because the Ecotron was designed to house entire model ecosystems², Naeem *et al.* were able to build multitrophic-level communities of 9, 15 and 31 species, with the species-poor communities representing subsets of the more diverse communities. This manipulation was cleverly designed so that the loss of diversity cut across each of four trophic levels: decomposers (earthworms and *Collembola*), primary producers (annual plants), herbivores (aphids, whiteflies, snails and slugs) and parasites attacking the herbivores (parasitic Hymenoptera), in much the same way that biodiversity on all trophic levels is being ravaged on a global scale by human activities. Even though each replicate community was confined to a 2 m × 2 m × 2 m chamber, species were selected so that reproducing populations could be accommodated within that space.

The experiment ran its course in a little over half a year, which allowed species to be added in a sensible order (for example parasitoids after their hosts had been established), and all plants to grow from seedlings to flowering adults. The variety of properties measured for each replicate ecosystem is impressive, and included community respiration, surface and underground decomposition, retention rates for nitrogen, phosphorus, potassium

and water, and plant productivity.

Among all these ecosystem processes, only productivity and respiration exhibited clear patterns with respect to the manipulation of biodiversity, with productivity increasing two- to threefold as biodiversity was increased two- to threefold. The mechanism underlying this trend may be an increase in light interception associated with plant canopies

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Inside the Ecotron: a technician takes light measurements.

that fill three-dimensional space more completely. Just as interesting as the connection between biodiversity and productivity suggested by this experiment is the absence of relationships between biodiversity and other ecosystem processes. It remains to be seen whether nutrient processing, for example, is generally insensitive to depletions in biodiversity.

What makes this study so effective is that the Ecotron has been used to address a question that has eluded field researchers. In particular, field studies of biodiversity have never been able to establish replicated communities that differ in species richness, yet are similar in all other major environmental variables. In addition, the few field experiments that adjust for environmental covariation when investigating the effects of biodiversity have had to restrict themselves to primary producers, leaving out the multitrophic dimension of the Ecotron work^{3,4}.

When one takes the results of Naeem *et al.* together with observations by Tilman and Downing³, and Ewel *et al.*⁴, it is becoming clear that the influence of biodiversity can be elegantly dissected through experimental manipulations. Perhaps more importantly, the results of these pioneering studies point towards the emergence of less productive

ecosystems¹, vulnerable to environmental perturbations³ and plagued by declining soil fertility⁴, upon dramatic reductions in biodiversity. Ironically, the consequences of our global biodiversity crisis are best illuminated to date by studies that involve experimental plots ranging in size from the Ecotron's 2-m-diameter plots, to at most 33-m-diameter plots⁴. Although ecology may indeed be turning into an "earth science"⁵, it is clear that there is still room for the small-scale laboratory or field experiment.

Unfortunately, the idea of using model systems such as those constructed by

Phil Heads

Naeem *et al.* has never taken hold in ecology in the way it has in molecular biology or cell and developmental biology. This is remarkable, given that several 'bottle experiments' or small 'microcosm experiments' are among ecology's most important and definitive contributions⁶⁻⁹ to science. Although the Ecotron and most other microcosm experiments can be labelled unnatural, that criticism misses the point — we do not use microcosms to mimic nature in its full complexity, but to speed up research and to gain control and replicate events in a way that is impossible in field studies. Thus, if one wanted to dispute the results and interpretation offered by Naeem *et al.*, they could duplicate the experimental conditions exactly,

and run a different set of experiments. Such repeatability is impossible in nature, which is why one never sees field experiments duplicated, and why one can never indisputably establish that some previous field experiment was erroneously interpreted. Of course, artificial ecosystems that do not exploit the power of replication and do not open themselves to critical scrutiny lose the one major advantage they have — which may explain why the privately funded Biosphere 2 project in the United States has received so little scientific support¹⁰.

Studies of microcosms in Ecotron chambers will never replace field experiments or exploration of naturally occurring patterns. But Ecotron-based research promises to provide an important counterpoint to the large-scale global monitoring and long-term data sets that are increasingly seen as necessary research tools

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for addressing global environmental concerns. Ecologists might be appalled by the price of a system such as the Ecotron, which cost the UK Natural Environment Research Council about £1 million to build, but such a facility is still cheap in comparison with the facilities upon which oceanography, astronomy and physics depend. If we had a dozen Ecotrons instead of just one, our ability to explore hypo-

theses regarding the consequences of global climate change, invasions by exotic species and habitat depletion would be staggeringly accelerated. One can only hope the vision behind the Ecotron is contagious. □

Peter Kareiva is in the Department of Zoology, University of Washington, Seattle, Washington 98195, USA.

SOLAR SYSTEM FORMATION

Celestial body-building

Paul Weissman

How does an interstellar nebula go about remaking itself into a planetary system? Starting with an immense, diffuse cloud of dust and gas, with a density of perhaps only tens of atoms per cubic centimetre, the cloud transforms itself into a compact system consisting of a central fusion-powered star and a collection of planets, satellites, asteroids and comets, in only 10

where gravitational instabilities cause them to collapse into larger bodies, several kilometres across. This is the typical size observed for cometary nuclei.

The result is not unlike two popular models of cometary nuclei proposed in the past decade. Bertram Donn and David Hughes suggested in 1985 that comets would be fractal assemblages of smaller

about $16 \times 8 \times 7$ km (refs 7, 8). But the resolution of the best images, about 80 m, was not quite good enough to provide an understanding of the underlying structure of the cometary nucleus.

Perhaps better evidence comes from the recently disrupted comet Shoemaker-Levy 9, which consisted of 21 individual nuclei when discovered⁹ in March 1993. The comet broke up when it passed within the Roche limit of Jupiter (the distance at which tidal forces can overcome a body's material strengths and self-gravity) in July 1992. Estimates of the sizes of the fragments range from as little as 50 m to about 5 km, although the best guess is probably about 1 km. This is somewhat larger than the primordial building blocks predicted by Weidenschilling. However, Weidenschilling points out that each of these is probably a 'clump' of many thousands of his initial bodies, and that individual bodies tens of metres across would be too small to be seen from Earth.

The best estimate of the sizes of the fragments may come when they smash into Jupiter at 60 km s^{-1} in July of this

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Rubble piles? Comet Shoemaker—Levy 9, photographed by the repaired Hubble Space Telescope in January 1994.

to 100 million years. Some of the key steps in that process are now outlined by Stuart Weidenschilling on page 721 of this issue¹.

A long-standing problem for Solar System theorists has been how submicrometre interstellar grains come together to form larger bodies. During the collapse of an interstellar cloud, the dynamical interaction between the dust and the gas causes solid grains of dust and ice to settle towards the central plane of the rotating nebula. Such dust disks are commonly observed around young protostars. Astronomers expected that as the density at the central plane grew, grains would coalesce into larger bodies because of their own self-gravity. However, the increased density near the plane results in turbulence, and that in turn halts the settling before the critical density for gravitational instability is obtained.

Weidenschilling's solution to this dilemma is a multi-step process, whereby dust and ice grains collide and slowly grow into larger bodies in the turbulent layer. As these first macroscopic bodies grow, they become less subject to the gas drag of the nebula. When they reach several tens of metres in diameter, they decouple from the gas and settle to the central plane

icy nuclei, extending down to microscopic assemblages of interstellar grains and ices². At about the same time, I proposed that many observed cometary phenomena such as splitting and outbursts could be explained if nuclei were 'primordial rubble piles' of smaller icy conglomerates created in the solar nebula³. What Weidenschilling has done is provide a physical mechanism for actually assembling such bodies with the required dimensions and physical attributes.

An additional feature of Weidenschilling's model is that the cometary nuclei form in clusters that may subsequently accrete to form even larger bodies, several hundreds of kilometres in diameter. The recent discovery of eight bodies, all about 100–250 km in diameter, in orbits beyond Neptune and Pluto, demonstrates that such large bodies did indeed grow in the outer reaches of the solar nebula^{4–6}. The largest 'comets' to grow in the outer nebula may be Pluto itself and its large satellite Charon.

Can Weidenschilling's proposal be confirmed by observations? Imaging of the nucleus of comet Halley in 1986 during the Giotto and Vega spacecraft flybys showed a highly irregular body with dimensions of

year. Since discovery, two of the initial fragments have faded from view, and two others have themselves split (see figure), so the size of the nuclei may in fact be substantially less than 1 km. Observations of many meteor flashes associated with each impact, in addition to the bright flash of the major fragment, would confirm Weidenschilling's belief that each large clump is accompanied by a swarm of smaller primordial fragments. Observers are being encouraged to look for such phenomena.

Another piece of evidence comes from chains of tightly packed, often overlapping craters seen on the surfaces of the icy galilean satellites. The discovery of Shoemaker-Levy 9 suggested that other comets passing through the Roche limit of Jupiter might break up and encounter one of Jupiter's icy satellites on their way out of the system¹⁰. The individual fragments would be like a line of machine-gun bullets lacing into the orbiting satellite. Given the observed sizes of the craters in the chains and the expected velocities for comets passing through the Jupiter system, the inferred dimensions of the individual impactors are again about a kilometre in diameter. This fits well with the observa-

tions of Shoemaker–Levy 9, but again is larger than Weidenschilling's prediction.

The obvious way to resolve the problem is to send a spacecraft to a typical comet nucleus and to orbit it, observing its structure and determining its mass to high accuracy. The European Space Agency is currently moving forward with plans for the Rosetta mission, which will do precisely that, along with carrying a sophisticated complement of compositional instruments to study the cosmochemical record bound up in the comets. Rosetta is currently planned to launch in 2003, with arrival at the comet in 2010.

NASA may join the comet hunt through its new Discovery programme of small, cheap interplanetary spacecraft. Missions currently under study include several that perform cometary flybys, and

one that could orbit a comet, as Rosetta will, although with a more modest scientific payload. The selection for NASA's next Discovery mission will take place late this year. □

Paul Weissman is in the Earth and Space Sciences Division of the Jet Propulsion Laboratory, Pasadena, California 91109, USA.

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MATERIALS SCIENCE

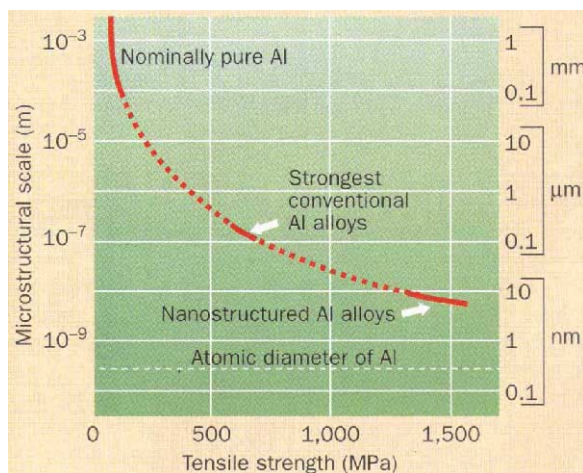
Nanostructure by nucleation

A. Lindsay Greer

IN the design of alloy microstructure, divergent trends are evident. For some applications, large single crystals are the ideal; for others, very fine-scale microstructures are desirable — results such as those of H. Gleiter¹ on condensation and compaction of clusters have shown that grain sizes of a few nanometres are possible. Methods of making nanostructured materials vary, but most involve compacting a fine powder. Circumventing this step, and producing nanocrystallites direct from the liquid, has already allowed a Japanese team headed by A. Inoue to produce exciting new part-crystalline, part-amorphous materials². Now comes a paper from R. Nagarajan and K. Chattopadhyay³ of Bangalore University, demonstrating that fully crystalline nanostructured intermetallics can be manufactured by a similar route, and opening up the possibility of a new family of alloy microstructures based on copious nucleation in the liquid state.

If a fine-scale microstructure is to be produced from a liquid, copious nucleation would seem to be the obvious mechanism. For the scale to be fine enough to be interesting, the nucleation must be homogeneous, or at least not dependent on added nucleants. In fact, it is only recently that it has been possible to exploit this route to

refinement⁴. The problem is that crystal growth releases heat, and the warming of the liquid stifles further nucleation. All is well if the crystal growth is sufficiently slow; this is just what is found in a glass,



One of the reasons for interest in nanostructured materials is readily seen by comparing strengths in aluminium alloys. The nanostructured alloys made by rapid quenching have remarkably high strengths². These materials have 20–25 volume per cent of crystalline aluminium particles in an amorphous matrix, the particle size and spacing being a few nanometres.

and crystallization of metallic glasses on heating is now well established as a route to nanostructured materials with excellent magnetic properties, both soft⁵ and hard⁶.

It should also be possible to make nanometre-scale microstructures by directly cooling the liquid, particularly on the margin of glass formation. The pioneering work in this field (recently reviewed²) has been by Inoue and col-

leagues, who have developed aluminium alloys (a typical composition, in atomic per cent, is $\text{Al}_{88}\text{Ni}_6\text{Ce}_2\text{Fe}_1$) with quite remarkable strengths, up to three times that of the best precipitation-hardened aluminium alloy (see figure). These have microstructures of aluminium particles, 3 to 10 nm in diameter, in a glassy matrix, and their strengths can at least partly be attributed to the crystals being simply too small to contain any dislocations.

The new material prepared by Nagarajan and Chattopadhyay is rapidly quenched near-equiatomic Ti–Ni (typically $\text{Ti}_{49}\text{Ni}_{50}\text{Si}_1$ in atomic per cent). One intermetallic phase, Ti_2Ni , nucleates as a nanodispersion in the liquid, the remainder of which then crystallizes to the compound TiNi around these particles. At the highest quenching rates used, the matrix grain size is of the order of one micrometre while the particles have a median size of about 10 nm.

With no amorphous matrix, the link with glass formation is less obvious than for Inoue's aluminium alloys. But the Ti–Ni system does show glass formation at somewhat higher titanium contents (60–70 atomic per cent Ti; ref. 7), and this reflects the same importance of stifling crystal growth. As Nagarajan and Chattopadhyay point out, the rapid quenching gives a kinetic competition between two crystalline phases in a highly undercooled liquid. The particulate phase must show fast nucleation and slow growth while the matrix phase must show slow nucleation and fast growth. They added silicon to influence the competition and ensure that the desired microstructure was obtained — silicon promotes nucleation of Ti_2Ni and may also hinder its growth.

The properties of the intermetallic nanostructured material have not yet been measured, but some indication of why they should be interesting can be gained from Inoue's work on the aluminium-based alloys, which can be regarded as nanometre-scale composites. The greatly enhanced yield stress shown in the figure would on its own be enough to make these materials remarkable — yet they also show good retention of strength at high temperatures, ductility, toughness, and an ability to be formed into useful bulk components. And related aluminium-based materials with icosahedral-phase particles can also show interesting magnetic properties².

The remaining drawback has been the amorphous phase, with its attendant prob-

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lems of stability. So the Indian team's work really represents a big advance. Clearly, rapid quenching near glass-forming compositions can produce not just partially but fully crystalline materials with fine-scale microstructures.

Until recently, the focus of interest was on metallic glasses themselves, and materials with some crystallinity were regarded as rejects. The realization that their properties can be outstanding, and the link with the current interest in nanostructured materials, has changed all that. The new results of Nagarajan and Chattopadhyay show that the method can

be applied to a wide range of particulate and matrix phases. The materials once thrown away by those looking for metallic glasses now prove that a previously unexploited processing route can produce nanometre-scale microstructures and exciting properties. □

A. Lindsay Greer, of the Department of Materials Science and Metallurgy, University of Cambridge, UK, is currently on leave at the Section d'Études de la Solidification, CEREM-DEM Centre d'Études Nucléaires de Grenoble, 85X, 38041 Grenoble cedex, France.

CHEMICAL SENSORS

Curling in the heat

David A. King

AN ingenious miniaturized heat sensor, shown here, has been developed by Jim Gimzewski and colleagues at IBM Rüschlikon specifically for studies of surface reactions¹. A spin-off of the scanning probe microscopes developed in the same laboratory by Nobel prize winners Rohrer and Binnig, the device is based on the

silicon levers used to detect atomic-scale protuberances on surfaces in the atomic force microscope.

The levers below are about 0.5 mm long and 1.5 μm thick; each is a tiny sliver of silicon coated with a 0.4- μm layer of aluminium. Small changes in temperature cause the lever to bend because of the bimetallic effect: the thermal expansion coefficient of aluminium is eight times that of silicon. The degree of bending is proportional to the heat flux dQ/dt absorbed by the lever, and is detected by measuring the change in direction of the reflected light beam, as shown schematically in the diagram. Radiation absorbed from the laser beam can itself be used as a means of calibrating the degree of bending by varying the laser power; a sensitivity of about 1 $\text{\AA}$ per nW absorbed radiation was obtained. In use as an atom probe, this type of lever detection system is capable of resolving movements of 0.1 $\text{\AA}$, which indicates the extraordinary power sensitivity of the device.

To illustrate its potential as a micro-

calorimeter for surface processes, the authors deposited a thin (0.04 μm) platinum film onto the lever, making a Si-Al-Pt sandwich. Under ultrahigh vacuum conditions, and starting with a clean platinum surface, the lever should bend when a dose of a reactive gas, such as O_2 , is introduced, because heat will be released in the exothermic process of chemisorption. Once adsorption is complete, the lever should return to its original position.

In their preliminary experiments, the vacuum was rather too poor for this to be done. Instead, a mixture of O_2 and H_2 was introduced into the reaction vessel, and the lever deflection recorded. Oscillations were observed, with a period of about 60 s, and attributed to the existence of self-sustaining chemical oscillations which have previously been reported for the H_2O formation reaction over platinum surfaces.

Clearly, one can foresee numerous applications for the device, perhaps in gas sensors, radiation detectors or quantitative calorimetry. A few years ago, I was one of a team who developed a quantitative microcalorimeter for studying the reactions between gases and single-crystal surfaces²⁻⁴. How does the new instrument compare? Our sensitivity is currently about 0.1 nJ (ref. 5) over a single-crystal sample area of $1.2 \times 10^{-5} \text{ m}^2$; the sandwich lever system is, say the authors, sensitive to about 1 pJ over a sample area of $1.4 \times 10^{-8} \text{ m}^2$.

Expressed as a sensitivity per unit area, there is not much to choose between them. But clearly the different sample sizes of the two instruments give rise to different advantages and disadvantages. The principal disadvantage of the smaller sample size is the severe difficulty in measuring the gas sticking probability, and hence the amount of gas adsorption, a problem readily surmounted in our system². A major advantage could be in differential calorimetry studies, with one lever calibrated against a second lever without a reactive coating, of heat changes due to phase transitions in adsorbed layers and at clean surfaces. One problem which will have to be dealt with is the influence of these changes on the surface stress at the reactive surface: this will also cause the lever to bend. Nevertheless, this clever device looks likely to resurface in a wide range of applications. □

David A. King is in the Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, UK.

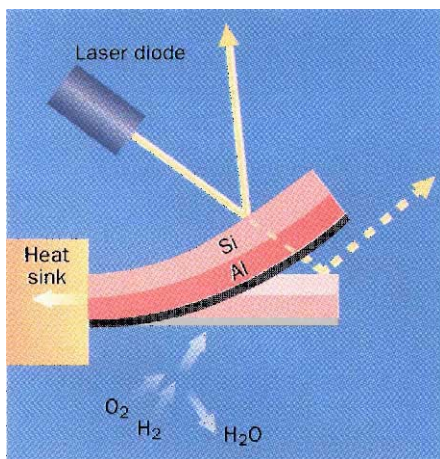


IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Top, measuring the bending of a bilayer lever caused by heat released in reaction on a platinum surface (grey). Bottom, an array of 10 such sensors.

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The eye in a twinkling

Richard Dawkins

THE creationist's favourite conundrum — "What is the use of half an eye?" — is a lightweight question, a doddle to answer ever since Darwin first anticipated and elegantly solved it in 1859. A more ponderous show of weight seems to lie behind the inevitable supplementary: "Speaking as a physicist [engineer etc.], I cannot believe that there has been enough time for an organ as complicated as the eye to have evolved from nothing". Both questions stem from what I have unkindly called *The Argument from Personal Incredulity*¹. Audiences nevertheless appreciate an answer and I have usually fallen back on the sheer magnitude of geological time. If one pace represents one century, the whole of time *anno Domini* is telescoped into a cricket pitch. But then, to reach the origin of multicellular animals, you'd have to slog it out all the way from New York to San Francisco.

It now appears that the shattering extent of geological time is a steamhammer to crack a peanut. Trudging from coast to coast dramatizes the time available for the evolution of the eye. But a study by Dan Nilsson and Susanne Pelger, published this week in *Proceedings of the Royal Society*², suggests that a ludicrously small fraction of that time would have amply sufficed. When one says 'the' eye, by the way, one implicitly means the vertebrate eye, but serviceable image-forming eyes have evolved between 40 and 60 times,

independently from scratch, in various invertebrate groups³. Among these 40-plus independent evolutions, at least nine distinct design principles have been discovered, including pinhole eyes, two kinds of camera lens eyes, curved-reflector ('Jodrell Bank') eyes, and several kinds of compound eyes⁴. Nilsson and Pelger have concentrated on camera eyes with refracting lenses, such as are well developed in vertebrates and octopuses.

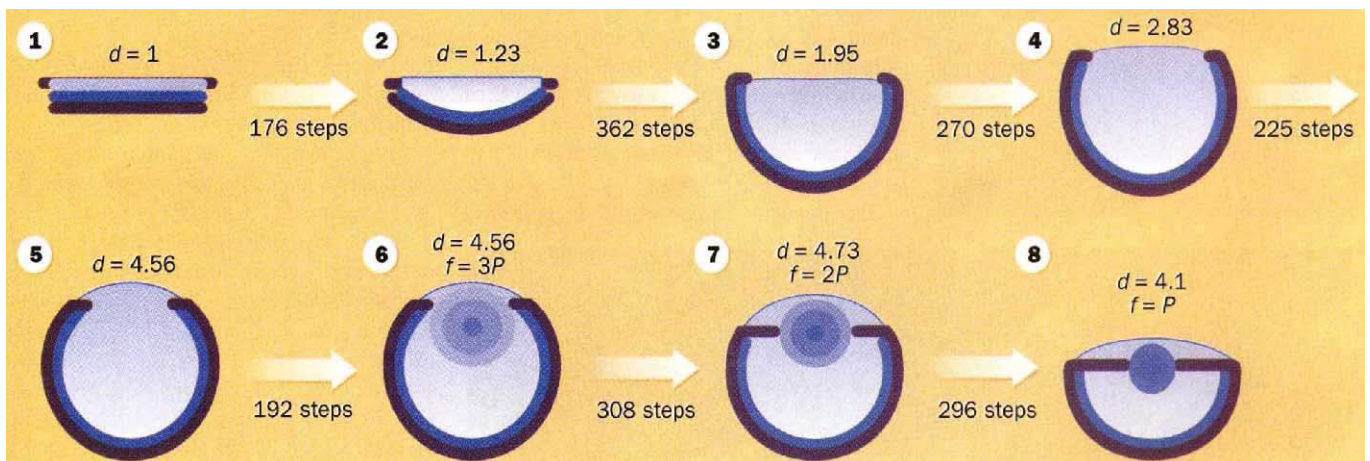
How do you set about estimating the time required for a given amount of evolutionary change? We have to make an assumption about the size of each step and it is sensible to express it as a proportion of an existing measurement (such an assumption underlay J. B. S. Haldane's definition of the Darwin as a logarithmic unit of evolutionary rate). Nilsson and Pelger used the number of successive changes of 1% as a unit for quantifying changes of anatomical quantities. If the change is all in one dimension it is then rather easy. In the unlikely event that natural selection favoured bird-of-paradise tails of ever-increasing length, how many steps would it take for the tail to evolve from one metre to one kilometre in length? A 1% increase in tail length would not be noticed by the casual birdspotter. Nevertheless, it takes surprisingly few steps to elongate the tail to 1 km — fewer than 700.

Elongating a tail from 1 m to 1 km is all

very well (and all very absurd), but how do you place the evolution of a camera eye on the same scale? The problem is that lots of things have to go on in lots of different parts of the eye, in parallel. Nilsson and Pelger's task was to set up computer models of evolving eyes to answer two questions. First, is there a smooth trajectory of change, from flat skin to full camera eye, such that every intermediate is an improvement? (Unlike human designers, natural selection can't go downhill, not even if there is a tempting higher hill the other side of the valley.) Second, how long would the necessary quantity of evolutionary change take?

Nilsson and Pelger worked at the level of tissue deformations, not the level of cellular biophysics. The existence of a light-sensitive cell was taken as given. Doubtless this could itself be the subject of a later simulation, in which the variables of interest would be molecular. Most of the popular scepticism about the evolution of the eye, however, centres on the tissue level: how could flat skin have invaginated into a smoothly circular cup; how could a lens have appeared at the right distance away from the retina, and with the right refractive index; and so on? Nilsson and Pelger also stopped short of simulating the variable iris and variable focusing, though their methods would lend themselves to these problems and I hope they'll tackle them in the future.

The beauty of simulating an eye as distinct from, say, the leg of a running cheetah, is that its quality can be easily measured, using elementary optics. The eye is represented as a two-dimensional



Stages in Nilsson and Pelger's model sequence of eye evolution. In the initial stage (1) the structure is a flat patch of light-sensitive cells sandwiched between a transparent protective layer and a layer of dark pigment. In stages 2 and 3 the photoreceptor layer and pigment layer (the retina) invaginates to form a hemisphere. The protective layer deepens to form a vitreous body which fills the cavity. The refractive index of the vitreous body is assumed to be 1.35, which is only slightly higher than that of water, and not enough to give the vitreous body any significant optical effect. In stages 4 and 5 the retina continues to grow, but without changing its radius of curvature. This causes a gradual shift from deepening of the retinal pit to constriction of the distal aperture. The aperture size in stage 5 was chosen to reflect the typical proportions in real eyes of this type. In stages 6–8 a graded-

index lens appears by a local increase in refractive index. The central refractive index of the lens grows from an initial 1.35 to 1.52 in the final stage. Simultaneously the lens changes shape from ellipsoid to spherical and moves to the centre of curvature of the retina. As the lens shrinks, a flat iris gradually forms by stretching of the original aperture. The focal length (f) of the lens gradually shortens, and in stage 8 it equals the distance to the retina (P), producing a sharply focused system. The relative change in receptor diameter, required to keep sensitivity constant throughout the sequence, is indicated by the normalized receptor diameter d. The anatomical change between model stages is given as the number of 1% modification steps. (Figure and caption modified from reference 2, with permission).

cross-section, and the computer can easily calculate its visual acuity — that is, spatial resolution — as a single real number. It would be much harder to come up with an equivalent numerical expression of the quality of a cheetah's leg or backbone. Nilsson and Pelger began with a flat retina, atop a flat pigment layer and surmounted by a flat, protective transparent layer (see figure). The transparent layer was allowed localized random mutations of its refractive index. They then let the model deform itself at random, constrained only by the requirement that any change must be only 1% bigger or smaller than what went before. And, of course, in order for a change to be accepted, it had to be an improvement on what went before.

The results were swift and decisive. A trajectory of steadily improving acuity led unhesitatingly from the flat beginning through a shallow cup to a steadily deepening cup. The transparent layer thickened to fill the cup and smoothly

curved its outer surface. And then, almost like a conjuring trick, a portion of this transparent filling condensed into a local, spherical subregion of higher refractive index — not uniformly higher, but a gradient of refractive index such that the spherical region functioned as an excellent graded-index lens. Best of all, the ratio of the focal length of the lens to its diameter settled down at a close approximation to Mattiessen's ratio, long known to be the ideal value for a graded-index lens.

Turning to the question of how long the evolution might have taken, Nilsson and Pelger had to make some plausible population-genetic assumptions. They chose values of heritability, coefficient of variation and intensity of selection from published observations from the field. Their guiding principle in choosing such numbers was pessimism. For each assumption they made, they wanted to err in the direction of overestimating the time taken for the eye to evolve. They even went so far as to assume that any new

generation differed in only one part of the eye: simultaneous changes in different parts of the eye, which would have speeded up evolution, were banned. But even with these conservative assumptions, the time taken to evolve a fish eye from flat skin was under 400,000 generations. Assuming typical generation times of one year for small animals, the time needed for the evolution of the eye, far from stretching credulity with its vastness, turns out to be too short for geologists to measure. It is a geological blink. □

Richard Dawkins is in the Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK.

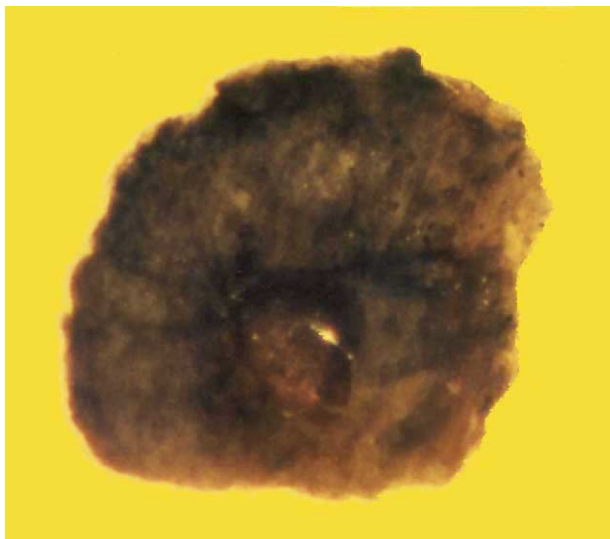
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METEORITICS

Fragments of history preserved

ABOUT 85 per cent of the meteorites observed to fall on the Earth are chondrites. Except for volatile elements such as hydrogen, helium and nitrogen, chondrites have elemental abundances similar to those of the Sun's photosphere; these meteorites preserve the bulk chemistry of the earliest solids to form in the cloud of gas and dust known as the solar nebula. Chondrites that were not greatly altered in their parent asteroids (by water, heat or shock) have heterogeneous mineral compositions and preserve the textures they inherited from the nebula, and typically contain submillimetre igneous spherules with solar compositions (known as chondrules), fine-grained dust of similar constitution, and grains of metallic iron–nickel and sulphide. Carbonaceous chondrites also contain abundant refractory inclusions (also known as Ca, Al-rich inclusions or CAIs), some of which have isotope anomalies suggesting the presence of pre-solar solids.

Chondrules are believed to have formed from clumps of precursor dust by transient heating events. Analyses of chondrule bulk compositions¹ suggest that several chemically discrete components were involved; one was rich in refractory lithophile elements and may have been derived from CAIs. The CAIs themselves also probably formed by high-temperature processes, some from



evaporative residues, others by condensation. The relationship between chondrules and CAIs is a long-standing puzzle: there are compound chondrules (made up of two chondrules stuck together) and there are compound CAIs, but until now there have been no known compound chondrule–CAI objects.

That is why the above chondrule from the Allende meteorite, described on page 723 of this issue² and pictured here about 40 times lifesize, is so important. For the first time we have a chondrule containing a relict (unmelted) precursor grain that appears to be a CAI fragment. The 200- μ m grain is spinel (MgAl_2O_4) containing several refractory silicate inclusions (including Al- and Ti-rich pyroxene) and platinum-group metal nuggets; all of

group metal nuggets; all of these phases have compositions within the ranges of those in CAIs in other carbonaceous chondrites.

Although Ca, Al-rich chondrules have been described before³, including some possessing fractionated rare-earth-element patterns similar to those in CAIs^{4,5}, these objects preserve only the chemical signatures of their CAI precursors. The chondrule described by Misawa and Fujita² preserves part of the CAI itself.

Taken to a plausible extreme, the evidence suggests that after CAIs formed in the nebula, some were broken by collisions and entrained in nebular dust before chondrule formation. Flash-heating of such clumps produced chondrules; dust clumps containing a high mass fraction of CAI fragments formed Ca, Al-rich chondrules. In the present case, the heating event was mild enough that part of the CAI survived.

Alan E. Rubin

Alan E. Rubin is at the Institute of Geophysics and Planetary Physics, University of California, Los Angeles, California 90024, USA.

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Reflections on a peptide

Leo Brady and Guy Dodson

EVOLUTION has ensured the almost exclusive occurrence of L-amino acids in naturally occurring proteins. Virtually all proteases therefore cleave peptide bonds between adjacent L-amino acids, and in consequence artificial proteins or peptides composed of D-amino acids are largely resistant to proteolytic breakdown. This resistance is attractive to drug designers,

two new elements: reverse synthesis and a change in chirality.

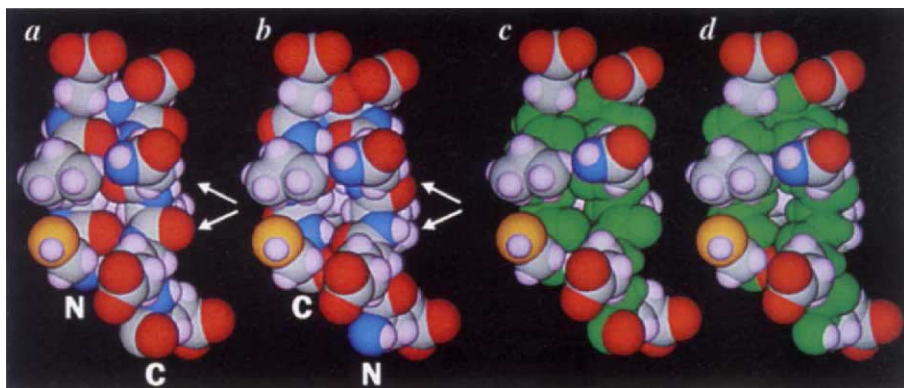
With the aim of overcoming proteolytic degradation and prolonging drug lifetime *in vivo*, Jameson *et al.* originally changed all naturally occurring amino acids in the CD4 hairpin loop for their D-enantiomers. The surface of this peptide was presumably a mirror image of the original pep-

bone are unchanged, the modelled surface is largely unaltered. Jameson *et al.* use this conclusion to explain the clear biological activity of their reverse D-peptide, which contrasts to the limited activity *in vivo* of its conventional all-L enantiomer (presumably a consequence of its susceptibility to proteolysis).

At first glance, reverse synthesis of all-D proteins might seem a way to produce a super-race of highly stable but conventionally active molecules. However, the approach has several limitations. For example, as the authors point out, secondary chiral centres in amino-acid side chains would need to retain their correct chirality; so peptides with threonine or isoleucine could present special problems. Equally, because of the reversal in the order of synthesis, sequences with proline would need to be avoided or treated with care. The prolyl ring joins the alpha carbon and peptide nitrogen, and in doing this constrains the Ramachandran angles. Reversing the peptide sense with D-proline will generate different local conformational constraints on the peptide. In the case described by Jameson *et al.*, the insertion of two prolines in the active reverse D-peptide must imply that these serve merely to separate the two strands at an appropriate distance and that the new peptide conformation fortunately does not compromise binding.

Prolines highlight a general drawback of the approach, namely that any interactions between side chain and main chain will be affected. Such interactions occur in most proteins; clearly they will be disturbed by the alterations along the main chain, and it is unlikely that an entire protein synthesized by the reverse-D method would fold correctly. The limitation arising from the replacement of each and every main-chain carbonyl with an amine group and amine with a carbonyl group matters less where they are in secondary structure, as main-chain/main-chain interactions within a protein are almost exclusively through complementary hydrogen bonds. The peptide segment chosen by Jameson *et al.* avoids all of the above pitfalls, clearly contributing to its success.

Protein-protein interactions are generally dictated by shapes and properties of molecular surfaces, and another of Jameson and colleagues' assumptions is that these surfaces are formed, for the most part, by side-chain atoms. This is not entirely true. Segments of altered main chain exposed at the surface may interact directly with another effector protein, and hence contribute to biological activity. One interpretation of the activity reported for the reverse-D CD4 analogue is that its receptor must interact mainly with the side chains of CD4 in the segment mimicked by the peptide. Reverse D-peptides may therefore be useful probes to disting-



Space-filling representations of the human equivalent of the peptide analogue used by Jameson *et al.* in their experiments on inhibiting experimental allergic encephalomyelitis. Coordinates have been extracted from the crystal structure of the amino-terminal domains of human CD4 (Ryu, S.-E. *et al.* *Nature* **348**, 419–426; 1990). Each atom is colour coded according to chemical type. The amino and carboxy termini are indicated. *a*, The L-amino-acid fragment which Jameson *et al.* mimicked in their synthetic analogue, without the connecting Pro-Gly-Pro-Cys motif which cyclizes the structure and evidently gives it a defined three-dimensional, active structure. *b*, The *in vivo* active reverse D-peptide analogue in which the direction of the now-D amino-acid peptide chain has been reversed. The side chains assume identical conformation and contacts (seen more easily in *c* and *d*, where the main chain is coloured uniformly green), but the main-chain carbonyl and amide amine groups switch position. Some of these changes are indicated by arrows. The positions of the side chains in these figures are not necessarily those of the peptide in solution or when complexed to the receptor. The figure also reveals how accessible some of the peptide main-chain atoms are, and so potentially available for interaction in any receptor complex.

but the exclusivity of biological systems for proteins made of L-amino acids means such proteins cannot interact with the mirror-image surfaces formed by enantiomeric proteins. Hence an all-D-amino-acid protein usually has no appropriate biological effects.

In an intriguing study published in this issue (*Nature* **368**, 744–746; 1994), Jameson *et al.* report an exception to this trend. This work is of more than passing interest, for the technique used to make the reverse D-amino-acid peptide may have other applications — provided the structural limitations of the method are considered and local folding is not compromised.

Jameson and colleagues' target is a hairpin loop from the CD4 receptor, a key protein in the immune system and one that is perturbed in experimental allergic encephalomyelitis (an autoimmune disease of rodents, on which the authors tested the effectiveness of their analogue). Although the design of peptide mimics of the biologically active surfaces of proteins is now commonplace, this new study combines

tide, however, and although resistant to degradation it did not have the appropriate biological effect. But if this all-D-amino-acid peptide is synthesized in reverse, with the carboxy terminus becoming the amino terminus (and vice versa), the resulting side-chain surface is an identical copy of its naturally occurring counterpart.

The net result of combining D-enantiomers and reverse synthesis is that the positions of carbonyl and amine groups in each amide bond are exchanged, while the position of side-chain groups at each alpha carbon is preserved (*a* and *b* in the figure). In the hairpin loop used by the authors, this alteration in the protein backbone is self compensating. Hydrogen-bond donors (amide amine groups) become hydrogen-bond acceptors (amide carbonyl groups) and vice versa: each pair is neatly reversed and the hydrogen-bonding pattern is preserved. The surface of the peptide is dominated by side-chain atoms (*c* and *d* in the figure), and if the positions relative to the back-

uish the relative involvement of main-chain and side-chain atoms in protein-protein interactions.

The technique described by Jameson and colleagues lies at the interface between synthetic chemistry and molecular biology, and largely stems from our ability to synthesize, analyse and visualize protein structures. Peptide synthetic chemistry can provide a far greater repertoire of fine protein manipulations than conventional site-directed mutagenesis and

molecular biology. In appropriate structural circumstances, there could be a considerable future in combining the two approaches. □

Guy Dodson is in the Department of Chemistry, University of York, York YO1 5DD, UK, and at NIMR, Mill Hill, London NW7 1AA, UK. Leo Brady, formerly also at York, is now in the Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, UK.

ENDOTHELIN-1

A matter of life and breath

Paul M. Vanhoutte

In a remarkable paper, published on page 703 of this issue¹, Kurihara *et al.* describe the consequences of disrupting the endothelin-1 (ET-1) gene in the embryonic stem cells of mice. Animals that do not produce the peptide exhibit severe morphological abnormalities of the craniofacial organs, which lead to the inability to breathe (and to live). Animals that produce decreased amounts surprisingly develop a mildly elevated blood pressure. These findings not only challenge the most commonly held view of the involvement of ET-1 in vascular control, but also show that the peptide has a hitherto unsuspected role in embryogenesis.

First, some history. In 1980 came the report that the endothelium has an obligatory part in the relaxations evoked by acetylcholine in isolated arteries². Endothelial cells inhibit the underlying vascular smooth muscle by secreting a short-lived vasodilator substance, originally termed endothelium-derived relaxing factor (EDRF), which turned out to be nitric oxide (NO; see refs 3, 4). Although endothelial cells secrete other vasodilators (see, for example, ref. 5), the demonstration that NO is indeed the main EDRF launched an explosion in knowledge centred on its ubiquitous function as a cellular messenger (reviewed in ref. 4).

Early on in the exploration of endothelium-dependent responses of isolated blood vessels, however, it became obvious that endothelial cells control the tone of the underlying vascular smooth muscle in a dual way⁶. Thus, particularly in veins, but also in coronary and cerebral arteries, different stimuli evoke endothelium-dependent contractions^{6,7}. Again, the transfer of some vasoactive substance(s) was involved — termed endothelium-derived contracting factor (EDCF) — and candidates for it included superoxide anions, thromboxane A₂ and endoperoxides.

Next, the finding that cultured endothelial cells can produce a potent vaso-

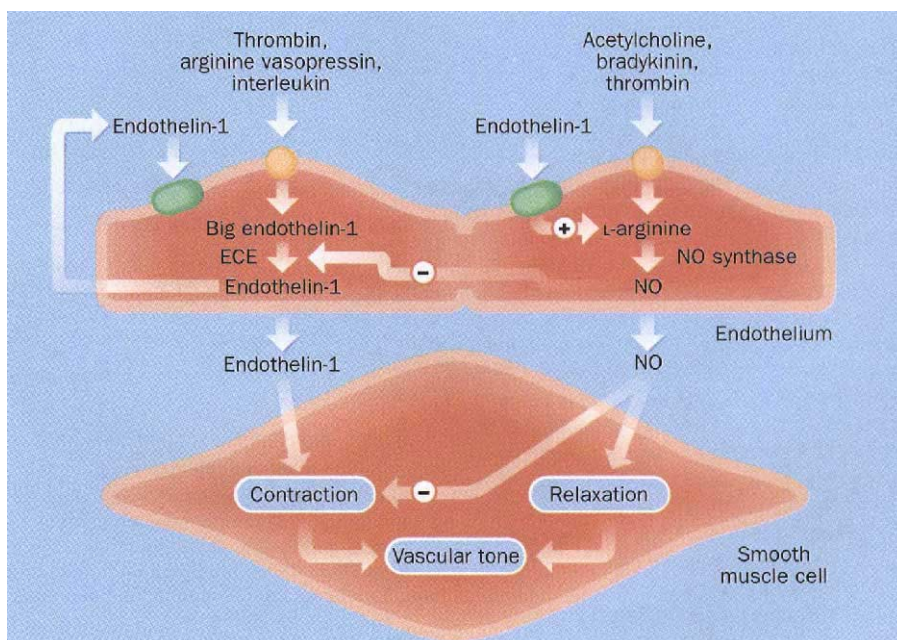
constrictor peptide⁸ prompted a line of research that culminated in the identification⁹ of a family of three such isopeptides called endothelins. As with NO, it soon became apparent that these peptides have widespread activity — they can be produced by a wide variety of cells, and through the activation of several receptor subtypes can affect the function of numerous tissues and organs^{10,11}. So far, ET-1 appears to be the most commonly occurring isopeptide, particularly in the human.

Endothelins are produced in excess in a variety of conditions characterized by tissue ischaemia, cell death or both — hence

the temptation to assume that they cause a number of pathologies accompanied by exaggerated vasoconstrictor tone. Indeed a simplistic view has emerged of an endothelial yin and yang, with a balance between the healthy mediator, NO (which not only causes vasodilation but also inhibits platelet aggregation and prevents adhesion of leukocytes), and the villain, endothelin (the vasoconstrictor *par excellence*). The idea finds support in the observations that NO prevents the formation of endothelin, and is the most powerful endogenous inhibitor known of the action of the peptide on vascular smooth muscle (see figure).

But the lack of sufficient data *in vivo* with selective antagonists of endothelin receptors warrants caution before accepting this view¹². In those conditions where over-production of endothelin has been reported, we do not know whether excess peptide contributes to the pathological process or whether it is an epiphenomenon, the last cry for help of the dying cells mistreated by disease (or the investigator?). Indeed, the new results¹ challenge the notion that endothelins are the bad guys of circulatory control. The diminished levels of ET-1, as seen in mice heterozygous for the gene, result in a modest increase in arterial blood pressure, rather than the expected reduction.

This result is at variance with the concept that ET-1 serves the sole purpose of augmenting peripheral resistance. Kuri-



Activation of endothelial cells by several vasoactive hormones and neurotransmitters increases production of the potent contractile peptide, endothelin-1 (ET-1), and of the potent relaxing factor, nitric oxide (NO). Endothelin-1 production results from the processing of its precursor big ET-1 by the endothelin-converting enzyme (ECE), whereas NO production results from conversion of L-arginine into L-citrulline by NO synthase. Nitric oxide curtails both the production and the action of ET-1. If it appears in the blood stream, ET-1 can stimulate production of endothelium-derived NO, which then counteracts its potent contractile activity. The interaction between ET-1 and NO produced by endothelial cells may be of key importance in the local regulation of vascular tone. (Reproduced from ref. 14 with permission.)

hara *et al.* remind the reader that, in the intact organism, the initial response to exogenous ET-1 is vasodilation⁹. They refer to work *in vitro* demonstrating that lower concentrations of the peptide cause endothelium-dependent relaxations because of the release of EDHF/NO. However, because inhibition of NO synthase caused comparable absolute increases in blood pressure in mice heterozygous for the ET-1 gene and in controls, Kurihara *et al.* conclude that a reduced release of NO does not explain the increase in arterial blood pressure seen when production of ET-1 is limited.

They are probably right, but only probably. The initial pressure was different in the two groups of mice, and it is difficult to compare the magnitude of vasoconstrictor responses in face of a changed baseline. For the same reason, further studies will be required, especially in the absence of proper concentration-response data, before one can fully accept their interpretation that the sensitivity of the endothelin receptors is the same in the two groups.

To come back to the issue of the reduction in endothelium-dependent effects of ET-1, as a contributor to the increased arterial pressure in mice heterozygous for the gene, one should keep in mind that endothelins not only cause release of NO but also that of other endothelium-derived vasodilators, such as prostacyclin and endothelium-derived hyperpolarizing factor (EDHF; ref. 13). To judge from experiments in rats, the release of EDHF by endothelin is more pronounced in young animals¹³, and could contribute to a depressor effect of the peptide in the very young mice used by Kurihara *et al.*

Overall, however, the experiments with the ET-1 heterozygotes are not only notable for their elegance but also for the important general message inherent in them. They show the need to concentrate on the effects of small amounts of endothelins (which have a depressor and vasodilator action in the intact organism), before considering the vasoconstrictor effects (which probably come into play only when their production is grossly accentuated). Endothelin-1 may not differ from catecholamines, serotonin or even vasopressin, all of which can have profound vasodilator effects that are often forgotten because of the overwhelming vasoconstriction that they cause at higher concentrations.

But the most extraordinary finding of Kurihara *et al.* is that absence of ET-1 in the embryo results in major abnormalities of the pharyngeal arch, and in the inability to breathe. This is so unexpected that it is, as yet, hard to comment on. The causal link between the absence of the peptide and the malformations finds support from results *in vitro*, where exogenous ET-1 has a positive effect on embryonic development. Endothelins have been considered

by some authors to stimulate cell growth (see ref. 11), but the new results promote ET-1 to the status of a key player in directing differentiation in a crucial (albeit localized) part of the body.

Moreover, the authors allude to the possible involvement of the peptide in the central-nervous control of breathing. Given accumulating evidence that the airway epithelium may be a major source of endothelin, we could be witnessing the emergence of endothelin as a general regulator of the formation and function of the respiratory system. In that regard, Kurihara *et al.* rightly note that their observations may provide a first glimmer of understanding of congenital anomalies such as Pierre-Robin and Treacher-Collins syndromes.

Finally, it seems that we also have the solution of a mystery — why nature has perpetuated the ability to produce a complex peptide such as endothelin, one which bears a strong resemblance to certain snake venoms and which is potentially so harmful if over-produced. Kurihara *et al.* now provide the answer: it is a matter of life and breath. □

Paul M. Vanhoutte is at the Institut de Recherches Internationales Servier, 6 place des Pléiades, 92415 Courbevoie Cedex, France.

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Corrections

■ The longest contiguous sequence of human DNA lodged in the data libraries is not the 73 kilobases of the β -globin region (News and Views, 3 March 1994; page 14). Rather that honour belongs to 180 kilobases encompassing the human retinoblastoma locus, deposited with GenBank by T. P. Dryja and colleagues (*Genomics* **17**, 535–543; 1993).

■ Discussion of the work of D. A. Maslov *et al.* (News and Views, 24 March 1994; page 288) should have also taken in a paper by L. F. Landweber and W. Gilbert that appeared in February (*Proc. natn. Acad. Sci. U.S.A.* **91**, 918–921; 1994). These authors also dealt with RNA editing in trypanosomatids, and came to broadly the same conclusions as Maslov *et al.*

DAEDALUS

Hidden motives

A PARASITE or disease organism lives by exploiting its host. A tapeworm steals your food, and an infection subverts your body chemistry. But they must do more than this: they have to propagate their kind onto further hosts. Accordingly, many parasites encourage their hosts to behave in ways that help the parasite to spread. Tuberculosis makes its victim cough and spit: this spreads the bacillus around, to be inhaled by other people. Hydrophobia goads a mad dog into biting other creatures, thus injecting them with the virus. A certain snail fluke causes the snail to climb grasses, so as to be more vulnerable to the birds that are the fluke's next host.

Musing on this principle, Daedalus began to wonder how deeply it influences human society. Perhaps compulsive hand-washing is triggered by an organism that is propagated on bars of soap. The custom of social kissing may be spread by an ingenious lip fungus. The bureaucracy that increasingly strangles whole societies may be an obsessive syndrome propagated by some malign organism that lurks in paper. It is clearly worthwhile to investigate just how, and how widely, parasites and disease organisms contrive to influence human behaviour.

So DREADCO's biochemists are studying various diseases which seem to modify the behaviour of their victims. Colds and influenza, for example, make the sufferer listless and unenterprising. The pathetic malaise invites others to crowd round with help and sympathy, and thus exposes them to the virus. The infection must release in the victim's bloodstream a sort of 'elixir of exhaustion'. Once identified and synthesized, it could prove a powerful antagonist to the obsessive drive of potential heart-attack victims, the hyperactivity of children and the fanaticism of zealots. A recent correspondent (D. F. Johnson *Nature* **368**, 93; 1994) suggests the use of intestinal worms to counter eating disorders. Daedalus hopes to achieve this result at second hand, so to speak. He notes that some worms make their hosts grow faster. Presumably the worm, needing its host to eat enthusiastically, puts out some natural appetite stimulant. If isolated in pure form, this stimulant might boost the growth of sheep and cows, and even cure anorexia nervosa. Similarly, the organisms of venereal diseases should encourage their hosts to propagate them in the obvious way. This encouragement might also be chemical. Daedalus is culturing these organisms in search of that unholy grail, an effective, natural aphrodisiac.

David Jones

Iron at the Galactic Centre

SIR — The neighbourhood of the Galactic Centre is a region of considerable astronomical interest. An innermost core of diameter ~ 300 pc emits high fluxes of radiation over a wide range of wavelengths. Recent X-ray studies of the Galactic Centre using the Japanese Astronomical satellite *Ginga* lend strong support to the idea that 10^3 – 10^4 supernovae have exploded in this region over the past 100,000 years^{1,2}. Furthermore, the ejecta from such explosions may have led to a localized enhancement of X-ray line radiation at 6.7 keV, corresponding to an increased concentration of iron². The relative abundance of iron in this region appears to be in excess of values in the Sun by a factor of about 2.

This observed excess of iron, if it arose

is also the dynamical timescale for the Galactic Centre plasma². Because an average type II supernova produces about 0.1 solar masses of iron, the mass of iron contained within, say, a sphere of radius $R \approx 150$ pc around the centre, is about 300 solar masses. The average column density of iron through this region is therefore $\sim 6 \times 10^{35} (3R/2) (4\pi R^3/3) \approx 10^{-6} \text{ g cm}^{-2}$.

These considerations can be seen in the light of the important implications of recent far-infrared observations made using balloons and satellites. A surprising discovery has been the depressed intensities found for both the C II (158 μm) and the N II (205 μm) lines at the precise location of the Galactic Centre, compared with the much stronger peaks of emission

desired extent, but would leave the CO line at 2.6 mm essentially untouched. Thus, the new far infrared data could be interpreted as further evidence for supernova-produced iron whiskers near the Galactic Centre.

N. C. Wickramasinghe
School of Mathematics,
University of Wales College of Cardiff,
Cardiff CF2 4AG, UK

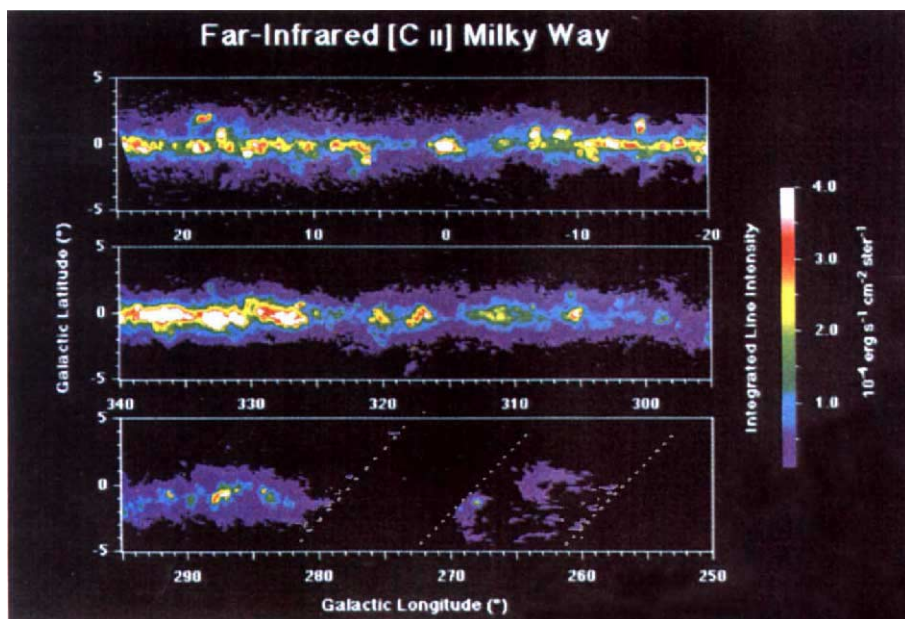
H. Okuda
Laboratory for Infrared Astronomy,
Institute of Space and Aeronautical
Studies, Sagami-hara, Kanagawa, Japan

Gelatinase A and APP

SIR — We previously reported¹ that APP, a precursor of the $\beta/\text{A4}$ amyloid peptide, has a proteinase inhibitor domain for gelatinase A, a member of the matrix metalloproteinase (MMP) family, and that the enzyme has α -secretase-like, βAP -hydrolytic activity. These facts suggest the possibility that gelatinase A may function in the metabolism of APP as α -secretase or βAP -degrading enzyme. Walsh *et al.* in Scientific Correspondence² reported that the rat adrenal pheochromocytoma cell line PC-12 does not secrete gelatinase A, and that addition of the metalloproteinase inhibitor TIMP-1, a glycoprotein of M_r 28,000, into the culture of PC-12 cells does not affect the secretion of APP. They therefore concluded that gelatinase A is unlikely to be α -secretase in these cells.

We have now carried out similar experiments using the same cell line but have obtained different results. Analysis of serum-free conditioned medium of PC-12 cells by substrate gel SDS-PAGE (zymography) shows that PC-12 cells secrete the latent proforms of gelatinases A and B (see figure). When the cells are induced by nerve growth factor to differentiate into neuronal cells, the secretion of both enzymes, especially gelatinase A, is markedly stimulated. This treatment stimulates not only the secretion of the proenzymes but also their conversion into active forms. The discrepancy between our results and those of Walsh *et al.*² may result from differences in experimental methods. Using northern blotting, we find that messenger RNAs encoding gelatinase A and its natural inhibitors, TIMP-1 and TIMP-2, are constitutively expressed in human brain tissue (data not shown), suggesting that gelatinase A may play a fundamental role in the central nervous system.

In agreement with Walsh *et al.*², we observed no significant effect of TIMP-1 on the APP secretion when it was added into the culture of PC-12 cells. But in PC-12 cells³ and human neuroglioma (H4)



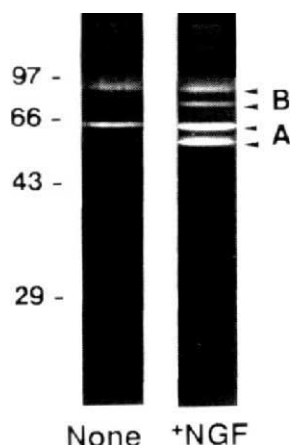
Map of the galactic plane in 158 μm C II line radiation contours.

from supernovae, is likely to be in the form of solid particles, probably as iron 'whiskers'^{3,4}. Such particles possess high values of the far infrared absorption coefficient that could lead to important observable effects⁵. The calculated mass absorption coefficient for cryogenic iron whiskers of radius 0.01 μm and of various lengths peaks at wavelengths in the range 100–300 μm , reaching maximum values of a few times $10^6 \text{ cm}^2 \text{ g}^{-1}$ (see ref. 5 for details of the calculations).

For such values of the absorption coefficient, and with reasonable assumptions about the energy density of the radiation field in the Galactic Centre region, the average time for supernova grains to be expelled due to radiation pressure from an inner core region of diameter ~ 300 pc is about 10^7 yr. This is well in excess of the 10^5 -yr timescale during which about 3,000 supernovae seem to have exploded, according to the recent X-ray data, which

that occur at galactic longitude = 330–340° (refs 6, 7). This situation also contrasts markedly with the data for the CO line at 2.6 mm, which shows its strongest intensity at the Galactic Centre itself. The figure shows this effect quite strikingly in the case of the C II emission contours from the galactic plane⁷. Iron particles of the kind thought to form in supernova explosions would clearly depress the strengths of both the C II and the N II lines to the

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Secretion of gelatinases A and B by PC-12 cells in the absence (none) and presence (+NGF) of nerve growth factor. Arrows A and B, gelatinases A and B, respectively. In each enzyme, the upper band is the proenzyme, and the lower is the mature enzyme. The levels of the proenzymes increased about 16-fold for gelatinase A and about 4-fold for gelatinase B by the NGF treatment.

METHODS. PC-12 cells were grown to semi-confluence in 10 ml RPMI-1640 medium supplemented with 10% horse serum and 5% FCS on collagen-coated 10-cm plastic dishes. The cultures were washed with serum-free RPMI medium and then incubated in the serum-free medium in the absence or presence of 50 ng ml⁻¹ NGF at 37 °C for 2 days. Resultant conditioned media were collected and concentrated 30-fold by ammonium sulphate precipitation, and then subjected to zymography on a gelatin-containing polyacrylamide gel as described previously⁵.

cells⁴, most of the mature membrane-bound APP is intracellularly cleaved by α -secretase in the trans-Golgi network or in a post-Golgi compartment rather than at the plasma membrane. Therefore, we expect that the addition of TIMP-1 into culture medium would not affect the intracellular action of α -secretase, even if gelatinase A is α -secretase. Taken together, these data show that the possibility that gelatinase A is α -type APP secretase cannot yet be excluded.

Kaoru Miyazaki

Kayano Funahashi

Makoto Umeda

Kihara Institute for Biological Research,
Yokohama City University,
2-120-3 Nakamura-cho, Minami-ku,
Yokohama 232, Japan

Atsuhisa Nakano

Department of Neurosurgery,
Hyogo College of Medicine,
1-1 Mukogawa-cho, Nishinomiya,
Hyogo 663, Japan

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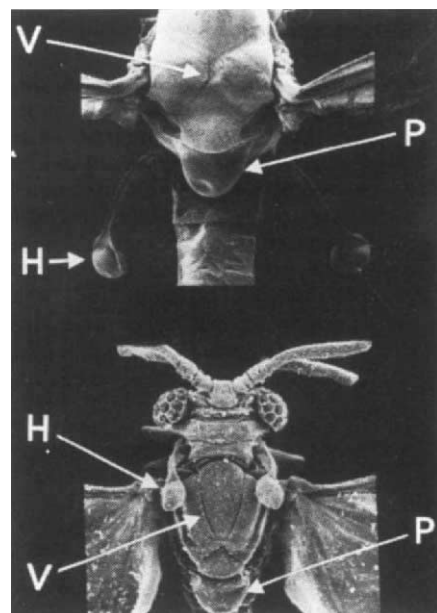
Insect homeotic transformation

SIR — Strepsiptera is a small order of insects with unusual morphological, physiological and behavioural characteristics. Molecular and morphological data support Strepsiptera as sister-group to Diptera. We suggest that a homeotic mutation resulting in ectopic expression of *Ultrabithorax* (*Ubx*) protein transformed the second thoracic segment (T2) to a metathorax and the third (T3) to a mesothorax in the ancestral strepsipteran lineage.

We determined the sequence of regions of nuclear 18S ribosomal DNA for representatives of Strepsiptera, all holometabolous insect orders, and outgroups. Details of data acquisition and phylogenetic analysis will be presented elsewhere. Relationships resulting from the molecular data are largely concordant with the most recent morphologically based phylogeny¹, except for the placement of Strepsiptera as sister-group to Diptera. We have found new morphological characters which support this hypothesis. Strepsiptera possess some ground-plan characters of supraordinal groups to which Diptera belong (loss of ovipositor, absence of labial endite lobes and spermatophore)². Moreover, in all male Strepsiptera, Mecoptera (scorpionflies) and basal Diptera³, abdominal segment 9 is enlarged and ring-like.

Similar morphological modifications of T2 and T3 occur on opposite segments in Diptera and Strepsiptera (see figure). We postulate that thoracic modifications derived in the immediate ancestor of these taxa were subsequently transformed to opposite thoraces in the ancestral strepsipteran lineage. This transformation might be caused by a homeotic mutation resulting in ectopic expression of *Ubx* in T2 and suppression in T3 in strepsipteran embryonic or larval stages. *Ubx* product in *Drosophila* is associated with specialization of T3 into a metathorax⁴. When suppressed by the mutations *bithorax* and *postbithorax*, the haltere is transformed into another full-sized wing and T3 is transformed into another mesothorax^{5,6}. Conversely, *Contrabithorax* mutations causing ectopic expression of *Ubx* in T2 result in a transformation of the T2 wing to a haltere (occasionally with some veins extant — as is found in Strepsiptera) and may transform T2 into a metathorax⁷.

Are Strepsiptera *Cbx Ubx/pbx bx*? Over-expression of *Ubx* in T2 and suppression in T3 would account for some of the unusual morphology of this group. We favour the transformation hypothesis because it more parsimoniously explains character distribution than to postulate parallelism, and despite potential lethality, has a plausible genetic basis. This



Scanning electron micrographs, dorsal view. Top, Diptera (*Tipula* sp., $\times 24$) and (Bottom) Strepsiptera (*Caenocholax fenyesi* Pierce, $\times 51$). H, halteres; V, V-shaped notal suture; P, expanded postnotum. In Diptera, V and P are on T2, H on T3; in Strepsiptera, V and P are on T3, H on T2. Halteres in Strepsiptera and Diptera are functionally identical (used for gyroscopic equilibrium during flight)⁸ and morphologically similar. In both groups the halteres are club shaped, filled with haemolymph, attached to the pleuron, and possess alula, tegula, and ventral chordotonal and sense organs². Because primitive wasps have a putatively homologous V-shaped suture on T2⁹, T2 placement of V and P and T3 placement of H are the primitive character states. Hence the hypothesized transformation would have occurred in the strepsipteran rather than the dipteran lineage.

hypothesis could be further tested by *in situ* hybridization of *Ubx* probes in strepsipteran embryos and instars. Strepsiptera may be one of the best examples of homeosis occurring in nature and causing drastic changes in the morphology of a group, which has led in part to its subsequent specialization and diversification.

Michael F. Whiting*

Ward C. Wheeler

Department of Invertebrates,
American Museum of Natural History,
New York, New York 10024, USA

*Also at: Department of Entomology, Cornell University, Ithaca, New York 14853, USA.

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Radiation and coral bleaching

SIR — Gleason and Wellington¹ ignore any effect that irradiance in the waveband 400–700 nm (photosynthetically active radiation, PAR) might have had on the bleaching they observed in coral colonies transplanted from 24 to 12 m depth. Under their experimental regime (use of cut-off filter), they subjected corals at each depth to two different irradiance profiles. The first, their 'UV present' specimens, received 100% ultraviolet radiation and 100% PAR for that depth; the second, 'UV absent' specimens, received 0% ultraviolet radiation and 92% PAR. At 12 m depth the transplanted corals subjected to the former profile bleached whereas ulcers on those subject to the latter did not. Gleason and Wellington unequivocally conclude that the bleaching response was a product of increased ultraviolet rather than enhanced PAR. They do not consider that the bleaching could also have been due to the 8% difference in PAR levels, or to a synergistic effect between the two types of radiation.

Undoubtedly the conclusion which they reached is the most appealing in the context of their paper, and may well be persuasive in logical terms. Scientifically, however, there are alternatives. Gleason and Wellington could have avoided the problem, which has been mentioned in earlier studies^{2,3}, by introducing a third treatment whereby transplanted colonies were placed under neutral density filters reducing the irradiance across the whole waveband (280–700 nm) by 8%. (Stainless steel wire mesh is a suitable material with a uniform attenuation at all ultraviolet and PAR wavelengths which can be tailored to provide appropriate attenuation). If thereby the ultraviolet irradiance had been reduced below any damage threshold at 12 m they could simply have incorporated an additional, shallower transplant depth.

Solar radiation is increasingly being recognized as a factor involved in bleaching of corals and other organisms that share a zooxanthellae symbiosis. Studies involving field manipulations⁴ and laboratory experiments⁵ at environmentally realistic irradiances have identified an involvement of solar ultraviolet, but to a subtle degree rather than visible bleaching. The assignment of roles to specific radiation wavelengths is complex because of changes in spectral quality and quantity under varying atmospheric conditions, and is further complicated by the underwater physical environment. Additional biological factors such as symbiotic interactions between the zooxanthellae and their animal hosts, and the need to consider the biological effectiveness (ac-

tion spectra) of different wavelengths (a consideration which Gleason and Wellington ignore in their dismissal of the waveband 280–300 nm), yet further add to the experimenter's burden. Although Gleason and Wellington have helpfully added to the general evidence implicating solar radiation in coral bleaching, they are not yet in the position to attribute it solely to ultraviolet radiation.

Richard P. Dunne

Department of Marine Sciences,
and Coastal Management
University of Newcastle,
Newcastle upon Tyne
NE1 7RU, UK

GLEASON AND WELLINGTON REPLY — Dunne suggests that we were premature in attributing the coral bleaching we observed in our experiments¹ to ultraviolet radiation because we did not take into account an 8% difference in PAR between treatments with and without ultraviolet; and because we did not consider potential synergistic effects of the two types of radiation. Although we admit that Dunne's first criticism identifies an imperfection in the experimental design, we believe it is highly improbable that this small difference between treatments caused the bleaching.

Coral colonies placed in 'UV present' and 'UV absent' treatments at 12 m depth were exposed to mean intensities of 106.07 W m^{-2} (s.e. = 6.65, $n = 17$ days, where daily values represent averages of scans obtained hourly between 1,000 and 1,300 h with a Li-Cor LI1800 spectroradiometer) and 97.58 W m^{-2} (8% reduction), respectively. Arguing that a difference of 8.49 W m^{-2} induced the bleaching in 'UV present' treatments assumes that *Montastrea annularis* colonies transplanted from 24 to 12 m depth were exposed to PAR intensities that were right at the boundary of their visible light tolerance. Our new (unpublished) data indicate this is probably not the case. Colonies of *M. annularis* moved from 24 m to less than 0.25 m depth, and when shielded from ultraviolet radiation showed no photoinhibition of photosynthesis following repeated exposure to PAR over a 12 h period. These results indicate that *M. annularis* can tolerate changes in PAR that are much more extreme than were introduced by our experimental manipulations.

Dunne's second criticism, that the bleaching may be the result of a synergistic effect between photosynthetically active and ultraviolet radiation while possible, also appears unlikely. In our experiment, coral colonies in 'UV present' treatments at 24 and 12 m depth were subjected to mean PAR intensities of 61.75 W m^{-2} (s.e., 5.15; range, 29.28–95.67 W m^{-2} ; $n = 18$ days) and 106.07 W m^{-2} (s.e., 6.65; range, 41.07–147.52 W m^{-2} ; $n = 17$ days),

respectively. Because of the overcast sky conditions and below-average water column clarity that prevailed during our field study in September–October 1991, PAR intensities experienced by colonies transplanted to 12 m depth were close to those observed at 24 m depth July 1991 (PAR mean = 85.21 W m^{-2} , s.e. = 7.26, range = 38.54–109.50 W m^{-2} ; $n = 11$ days). Thus, *M. annularis* colonies transplanted from 24 to 12 m depth were not exposed to radical increases in PAR. Further, the 24.5% enhancement in daily mean PAR intensities observed between 24 m depth in July 1991 and 12 m depth in September–October 1991 compares to increases of 37.2% for ultraviolet-A (320–400 nm), 220.0% for ultraviolet-B (300–320 nm) for the same depths and time periods. The biological action spectra for both DNA⁶ and plant responses⁷ is greatest, by several orders of magnitude, at wavelengths below 330 nm, making ultraviolet a more likely cause of the coral bleaching.

In summary, while we agree that Dunne has revealed a potential problem in the experimental design, the available evidence provides strong support for our conclusion that increases in ultraviolet radiation that result from rapid changes in water column conditions may be an important parameter contributing to coral bleaching events. We stand by that conclusion.

Daniel F. Gleason

Gerard M. Wellington
Program in Evolutionary
Biology and Ecology
Department of Biology,
University of Houston,
Houston,
Texas 77204–5513, USA

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Longest read?

SIR — If the statistical methods of Gott (*Nature* **363**, 315–319; 1993) for predicting future longevity on the basis of past longevity are applicable to all events, does this mean that there is a 95% probability we will be reading correspondence relating to the original article for the next 30.6 years?

Guy Hewlett

Bayer Pharmaceutical Research Centre,
Institut für Virologie,
42096 Wuppertal,
Germany

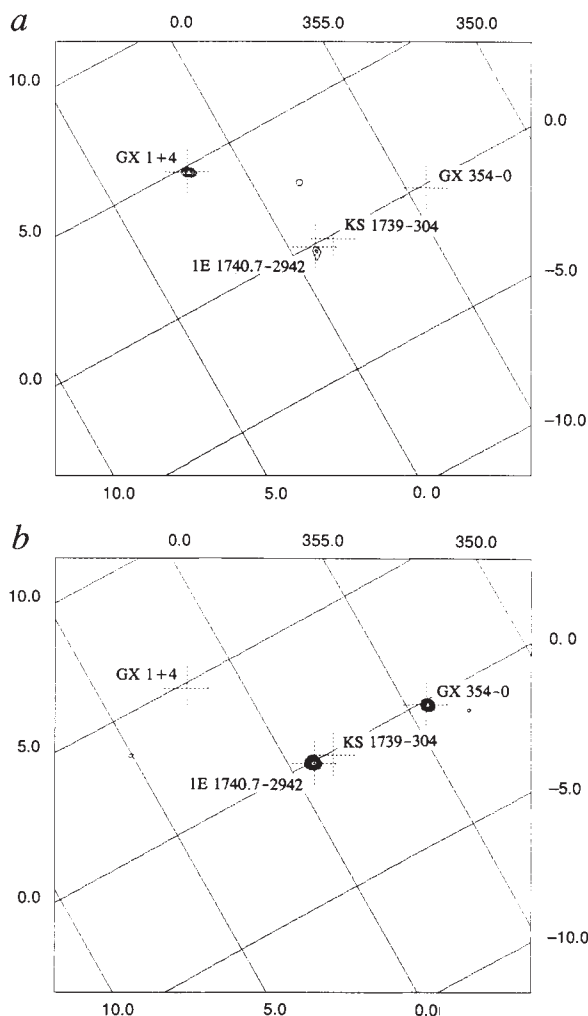
Earth occultation or coded mask?

SIR — Zhang *et al.*¹ argue that the Earth occultation imaging technique, as used by the BATSE experiment aboard the Compton Gamma-Ray Observatory, holds great promise for long-term, all-sky monitoring of hard X-ray sources, and appears particularly advantageous for space-born applications, since it does not require the use of a “massive” occulting mask which can limit the field of view of high-energy imaging experiments. To demonstrate the capabilities of such a method, Zhang *et al.* produce images of selected wide fields of the sky, including four images of the region containing the Galactic Centre (GC), taken at different times in the spring of 1992. A comparison between the last two BATSE GC images and nearly contemporaneous images obtained by the French coded aperture telescope SIGMA² aboard the Russian spacecraft GRANAT raises some doubt about the accuracy of the Earth occultation imaging technique where X-ray sources are tightly clustered.

Since March 1990, SIGMA³ has provided wide-field sky images in the hard X-ray and soft γ -ray domain, with an angular resolution of about 15 arcmin. Its coded mask, whose mass (including the support structure) is less than 10% of the total telescope mass, allows a $16.9^\circ \times 18.0^\circ$ field of view, contrary to the information reported in Table 1 of ref. 1.

The hard X-ray image of the GC region presented in part *a* of the figure combines the data recorded in the 50–100-keV band during two observations made on 17–18 and 18–19, February 1992, the same energy range and in coincidence with the 16–20 February BATSE image. Our map reveals two hard emission excesses above the instrument-detection limit, clearly identified with 1E 1740.7–2942 and GX 1+4. This latter source, which underwent a dramatic outburst on 17–18 February 1992 (ref. 4) and then dominated the GC region over the two days covered by the SIGMA observation

in the 50–100-keV band, is absent in the related BATSE image, where the only apparent excess concerns 1E 1740.7–2942, which was in a relatively low state at that time^{2,4}. The other SIGMA GC image (*b* in the figure) groups three observations performed on 1–2, 2–3 and 4–5 March 1992, which are mainly contemporaneous with the 2–6 March BATSE GC image. Although the two strong excesses detected in the coded mask image are unambiguously identified with GX 354–0 and 1E 1740.7–2942, the Earth occultation technique encounters severe difficulties in disentangling the field. Indeed, the BATSE image suggests a possible contribution of KS 1739–304, even when no hard emission from this latter source was detected by SIGMA^{2,5} (No previous marginal SIGMA detection of KS 1739–304 was claimed by Churazov *et al.*⁶, contrary to the information reported in ref. 1.)



Contour map in galactic coordinates of the GC field, derived in the 50–100-keV band from the data collected during the 17–19 February (*a*) and 1–5 March (*b*) SIGMA GC observations. The contour levels are in units of standard deviations (σ) over the background mean, starting from 3.5σ and further logarithmically disposed at $4.2, 5.0, 5.9, 7.1, 8.4$ and 10.0σ . In *a*, the GX 1+4 versus 1E 1740.7–2942 SIGMA count ratio is 2.3 ± 0.7 , while the GX “354–0” versus 1E 1740.7–2942 SIGMA count ratio is 1.01 ± 0.16 in *b*.

In consequence, although the Earth occultation imaging technique might appear attractive for all-sky routine survey of the hard X-ray sky, it should be used with great caution for long-term monitoring of hard sources, especially in crowded fields. Moreover, even in the case of the all-sky monitoring task, the limitations of such a technique in terms of source location accuracy may prevent further identification of rapidly varying, hard transient sources. This problem is illustrated by the case of GRS 1716–249, the X-ray nova in Ophiuchus, whose discovery was simultaneously reported by SIGMA⁷ and BATSE⁸, but whose radio, optical and X-ray counterparts were all discovered^{9–11} only because of the small error circle provided at hard X-ray energies by the coded-mask experiment.

Bertrand Cordier, Jacques Paul
DAPHNIA Service d'Astrophysique,
Centre d'Etudes de Saclay,
91191 Gif-sur-Yvette Cedex, France
Pierre Mandrou,
Jean-Pierre Roques
Centre d'Etude Spatiale des
Rayonnements,
9, Avenue du Colonel Roche,
BP 4346, 31029 Toulouse Cedex, France

ZHANG ET AL. REPLY — We are aware of the outstanding scientific observations in high-energy astronomy that are being accomplished by the SIGMA–GRANAT experiment, and we did not mean to imply that the Earth occultation technique can or should take the place of higher-resolution coded-mask imagers. Instead we consider Earth occultation imaging as a new and complementary technique for hard X-ray and γ -ray astronomy. We maintain that its primary advantages are its very wide field (or all-sky) capabilities and its relatively simple flight hardware, as described in our paper¹.

As pointed out by Cordier *et al.*, it is evident that the two nearby sources, 1E 1740.7–2942 and KS 1739–304, are not resolved by the preliminary Earth occultation images shown in our paper. We regret the erroneous reference for the transient source KS 1739–304 in the Galactic Centre region.

Shuang-Nan Zhang, Gerald J. Fishman,
B. Alan Harmon & William S. Paciesas
NASA Marshall Space Flight Center,
Alabama 35812, USA

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Writing up the past

John Cairns

The Strands of Life: The Science of DNA and the Art of Education. By Robert L. Sinsheimer. University of California Press: 1994. Pp. 318. \$30.

As Soutar wrote in *The Diaries of a Dying Man*, "We wish to be remembered even when none remain who looked upon our face; and, having gone from all touch, we trust that memory may, as it were, keep our unseen presence within the borders of day". Couple that with the chance of being paid to write your autobiography and the urge becomes irresistible.

The Sloan Foundation has commissioned autobiographies from several scientists in the hope of encouraging public understanding of science, and Sinsheimer's is the latest in the series. He was trained in physics and chemistry at the Massachusetts Institute of Technology (MIT) and spent the war years working on radar. When the war ended, he returned to MIT and worked on developing ways of measuring the absorption spectra of nucleic acids. As this was before the structure of DNA had been worked out, he was from the outset in a perfect position to record the momentous discoveries of molecular biology. After MIT, he went to Iowa State College and then to the California Institute of Technology, where he studied the small bacteriophage ϕ X174. Finally, after a few years as chairman of biology at Caltech, he became chancellor of the riot-torn Santa Cruz campus of the University of California and was, for a while, an outspoken opponent of genetic engineering. Undoubtedly he has lived in exciting times.

His story seems to have been a long series of successes. As he himself says, he was marked from the beginning as having considerable talents. Schoolwork, he writes, was ridiculously easy. In his first school, he was top even though he was the youngest in the class, and he was the highest-ranking male in his high-school graduating class. As the result of his training at MIT, his approach to biology has been "more quantitative, more analytically rigorous, more unrelentingly reductionist, perhaps more imaginative as to the ever-expanding potentials of ever-new techniques". In the physics department at Iowa State College, he studied the frequency of dinucleotides in DNA and claims to have been considering the possibility that DNA might have two strands held together by base-pairing before the structure had been published.

Sinsheimer once wrote that it was Max Delbrück who suggested that he should work on ϕ X174, and this was to be his major contribution to science. The virus has several claims to fame. It provided the first example of a single-stranded DNA molecule. Furthermore, the molecule is in the form of a circle. Sinsheimer says single-strandedness and circularity posed a conundrum. "If Watson and Crick were right, how could this DNA repro-



Sinsheimer in the lab at Iowa State College, 1951.

duce or even code?" He then goes on to say that the DNA was eventually shown to be converted to a double-stranded molecule from which single strands are made and packaged into virus particles. The DNA of ϕ X174 hit the news when Goulian, Kornberg and Sinsheimer reported that they had succeeded in replicating it *in vitro*, and this achievement was given a lot of publicity as a way of defending the National Institutes of Health from political attacks. Finally, the entire sequence of ϕ X174 DNA was determined by Sanger and this showed, among other things, that two genes that Sinsheimer's group had had difficulty in mapping were examples of a new phenomenon, overlapping genes.

Perhaps because this is an autobiography rather than a history of science, Sinsheimer does not mention other people's contributions to the story. Luckily, I

am not subject to this restraint. The Tessmans were, I think, the first to suggest that viruses such as ϕ X174 contain single-stranded DNA; Jacob, Wollman and Hayes were the first to suggest that some DNA molecules could be circular; many features of ϕ X174 replication, including the idea of a 'rolling circle', were worked out by Gilbert and Dressler; and others had already reported the preparation of functional transforming DNA and infectious RNA *in vitro*.

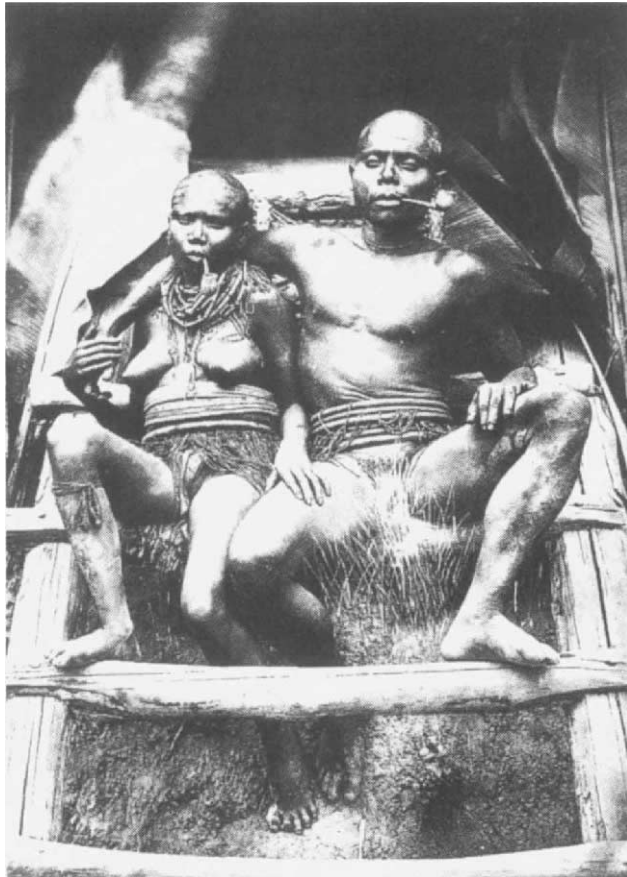
At the age of 57, Sinsheimer became chancellor at Santa Cruz, and roughly half of his autobiography is concerned with the ten years he spent there. To become head of a bankrupt university that has got through three heads in three years is to undertake a difficult assignment (this is the occasion for the only joke in the book,

which is his description of meeting a student who said she made a habit of coming to each year's reception for freshmen because she liked meeting the new chancellor). I know from personal experience how hard it is to convey to others the full horror of situations like this. Outsiders cannot be expected to be interested in accounts of life on the cliff edge of insolvency. So I will not comment on his chancellorship at Santa Cruz, poised on the San Andreas fault. More accessible are his accounts of trying to raise support for the construction of a very large telescope and, when that failed, trying to make Santa Cruz the centre for the effort to sequence the human genome. In each case, the foundations that might have supported these ventures did not want to be involved, perhaps because he was not backed up by the other leaders within the University of California. But he must feel some satisfaction from knowing that each project is now under

way, in an even more expensive form than he had proposed. Finally, in the last chapter, which takes its title "We Happy Few" from Shakespeare's *Henry V*, Sinsheimer discusses what it is about science that separates it from other human endeavours and assesses his own contributions. The subtitle is "Ending Well".

Sinsheimer's autobiography made me realize how hard life is for those who believe they are, and always have to be, the best at everything. Perhaps he should have followed the example of Henry V, who spent his youth in reckless dissipation so that the world would not expect too much of him when he moved from being prince to king. □

John Cairns is in the Department of Clinical Trials, Harkness Building, Radcliffe Infirmary, Oxford OX2 6HE, UK.



A SMOKE on the porch — Maia Biala, the Chief of Rutland Islands and his wife on the steps of their hut as photographed by G. E. Dobson in 1872. Taken from *Anthropology and Photography, 1860–1920* edited by Elizabeth Edwards. Introductory essays and historical and theoretical perspectives precede a collection of over 150 fascinating photographs focusing on British anthropology. Now published in paperback by Yale University Press, £14.95.

Global supermetaphors

David Edge

Metaman: The Merging of Humans and Machines into a Global Superorganism. By Gregory Stock. Simon and Schuster: 1993. Pp. 365. \$24.

THE day England won the soccer World Cup, I was a guest of Magnus Pyke, the eccentric popularizer of science. We were touring his estate and came upon his croquet lawn — a large expanse of turf encircled by a narrow, thinly stocked flowerbed. “You know my style, Edge”, Magnus said, with a grand sweep of his arm: “the vast generalization with a few trimmings.”

No one could accuse Gregory Stock of neglecting the flowerbeds: whereas his lawn (omitting the figures and spare space, and allowing for differing type-sizes) covers about 130 pages of written text, his glossary and notes occupy 100 — a pretty *bricolage* of cuttings (assiduously culled, mainly, from recent issues of *Nature*, *Science*, *Scientific American*, the *New York Times* and their like), all set in a mulch of degutted key texts and reference encyclopaedia.

The lawn’s the thing, however. What is

Stock’s vast generalization? To quote the dust cover: “that human society has become an immense living being — a global superorganism in which we humans, knitted together by our modern technology and communication, are like cells in an animal’s body”; that this is “more than a metaphor: it is an actual living creature”; and “convincingly arguing” that “present-day problems . . . are the ‘birth pains’ of Metaman”. In other words, Stock propounds a new vision. How his superorganism relates to, and differs from, earlier versions (such as Teilhard de Chardin’s ‘noosphere’, or Gaia) is left unclear; and, while much of what Stock says is consistent with sociological analysis of the institutionalization of modern science and technology, and anthropological discussions of culture, these powerful and well established bodies of theory and scholarship yield no cuttings for the flowerbeds. Stock’s vision is essentially a restatement of technological and evolutionary optimism, yet his chosen form is apocalyptic: he presents a vision of hidden forces with which we must contend but of which we are unaware, sweeping us remorselessly on. And he necessarily falls

into the traps of the genre. Three are worth mentioning here.

First, apocalyptic literature walks a tightrope: it must present a picture that is sufficiently dramatic to attract attention, but not so predetermined as to encourage inaction. If these things are going to occur anyway, why not just let it all happen? Like all such prophets, Stock is equivocal: his predictions often sound like hard technological determinism: “Some social changes are the inevitable consequence of these larger forces . . . beyond our control”, “the unavoidable product of . . . technological advance”. But the sting is usually drawn: “Such possibilities . . . are *almost* inevitable extrapolations of the scientific and technological advances of recent decades”, “*largely* not a matter of choice” [italics added]. This tension is not resolved: the reader is often left unclear as to which changes are “inevitable” and which are “the product of modifiable human influences”.

Second, the pattern is assembled without any explicit, critical methodology: it’s just put together (mainly out of scientific bits); there are no criteria by which to assess whether it is being done ‘better’ or ‘worse’, ‘right’ or ‘wrong’. Tricky issues, contentious judgements and political disputes are all discreetly wrapped up with a convenient citation, so that the overall optimism is not clouded. Controversy and conflict are glossed over: these nasty weeds are just birth pains, which we can expect eventually to fade away. No underlying theory guides this process of patterning. It’s simply a matter of “here’s what I see — don’t you see it too?” The reader must accept the author’s authority.

Third, propounding metaphors is a dangerous business, especially when one hints that they are literally true. Stock claims that Metaman has “the functional equivalent of a nervous system”, although this “does not *necessarily* mean that [it] is conscious”; earlier he states that “Metaman . . . is aware of the crucial aspects of its environment and is responding to them in its own self-interest. This ‘awareness’ does not require what we think of as consciousness, but merely the capacity to interpret sensory input.” Luckily, there is a simple word to describe such claims: nonsense. But any metaphor, by definition, is literally absurd: perhaps Metaminds are almost inevitably drawn to propound them.

Stock claims that his metaphor “helps to make sense of the underlying forces shaping our world”. But I doubt whether any readers will find substantial novelty here (especially if they are familiar with recent scholarship in the social sciences). I also doubt (*contra* Gaia) whether Metaman suggests any scientific predictions or conceptualizations that lend themselves to experimental elaboration. Is Stock, then, offering us a religious worldview? In a

way, yes: but this new myth contains no fragments of sharp, particular pain or joy — and does not therefore offer individuals any religious purchase, no point of passage from the exterior and mundane to the inner and spiritual. This book poses an age-old vast generalization: why do humans crave for an abstract structure of nonsensical claims, apparently validated by some higher authority, within which to locate their lives and aspirations? Why, indeed: Anyone for croquet? □

David Edge, reader emeritus at the University of Edinburgh, is at 25 Gilmour Road, Edinburgh EH16 5NS, UK.

Darwin here, there and everywhere

Nicholas Mackintosh

The Nature of Knowledge: Concerning Adaptations, Instinct and the Evolution of Intelligence. By Henry Plotkin. *Allen/Lane Harvard University Press* (as *Darwin Machines and the Nature of Knowledge*): 1994. Pp. 269. £20, \$24.95.

IMAGINE the following improbable scenario. Charles Darwin, is as we all know, a natural historian, but his interests are not confined to geology or the species question. Instead, he is drawn to the study of animal behaviour, and he is struck by the marvellous ways in which not only instinctive but also learned behaviour is so nicely attuned to an animal's needs. Fifty years before Edward Thorndike, he begins to investigate how cats and dogs learn by trial and error, how successful actions are repeated and unsuccessful attempts to solve a problem are eventually discarded. A simple theory occurs to him: animals in novel situations generate a wide variety of novel actions; some of the actions achieve nothing and, although perhaps repeated a few times, are eventually dropped; others, equally blind and random, however, happen to solve the problem; they are 'stamped in' or strengthened, and so repeated on future occasions. Not only has he anticipated Thorndike's "law of effect"; according to Henry Plotkin, he has discovered the principle of evolution by natural selection, and might have published his findings in a book entitled *The Origins of Behaviours by Means of Internal Selection or the Preservation of Favoured Acts in the Struggle for Being Remembered*.

Many readers may find this a surprising, even bizarre, claim. For Plotkin, however, it is no more than a recognition of the power and generality of the principle of evolution by natural selection. We all (well, almost all) accept that it explains the origin of species and much else about

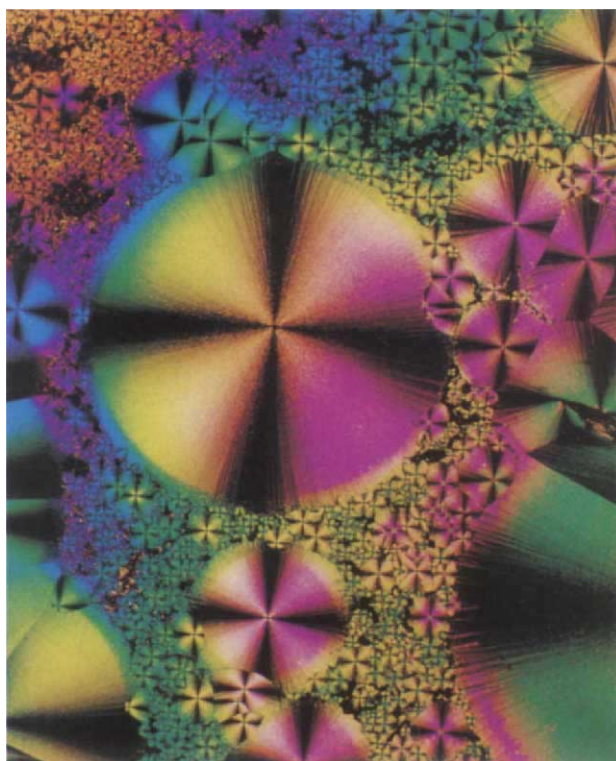
their function, structure and even, in the case of animals, behaviour. And, as we all know, this was the application of the principle that Darwin happened to chance upon. But properly understood, Plotkin argues, it is an entirely general principle, which can be instantiated by a variety of mechanisms and which serves to explain all manner of change. We see evolution by natural selection wherever there is some source of variation, and where some of the variants are more successful than others in passing a test imposed by a selection device and where these successful variants are able to transmit some of their features to their successors. Any systems that generates, tests and regenerates is probably instantiating this principle.

Plotkin's thesis, as he is the first to remind us, is not novel. Huxley, and Darwin himself, saw something of the generality of Darwin's discovery. Macfarlane Burnet's *Clonal Selection Theory of Acquired Immunity* persuaded immunologists that the immune system operated by the generation of a great diversity of lymphocytes, some of which happen to pass a test by fitting an invading antigen and are then clonally reproduced. Many psychologists, most notably B. F. Skinner, have pointed to the analogy between natural selection and the law of effect; thanks to Gerald Edelman, that analogy has been extrapolated to the neural mechanisms underlying all forms of learning. Karl Popper's account of the history of scientific knowledge as conjecture and refutation explicitly relies on the Darwinian analogy.

Plotkin is a psychologist and his book places most emphasis on learning or the acquisition of knowledge and the cultural transmission of that knowledge. It is an extended essay on 'evolutionary epistemology', a phrase coined by D. T. Campbell and rightly seen by Plotkin as a barrier to understanding. Indeed, one of this book's great virtues is that Plotkin writes incomparably more clearly than most others who have ventured into these fields. His exposition, even of complex issues, is beautifully lucid, his arguments well thought through and his illustrations apt. The book can be firmly recommended to anyone who has grappled with, say, Edelman's *Neural Darwinism* and come away defeated, but with a nagging worry that he or she might be missing out on some important ideas. Its very lucidity, however, may leave some readers less convinced.

What do we gain by pointing to these sorts of analogies between the evolution of species, the acquisition of knowledge and human culture? For Plotkin, the answer seems self-evident: "one of the objectives of science is to explain more and more with less and less.... Scientists hope that ever-greater numbers of phenomena in the world can be understood by the smallest possible number of scientific laws or principles." Well, I suppose so, although the history of psychology might serve to remind Plotkin that overarching theories are as often an impediment as a spur to good science. And while we rightly value some analogies because they point to underlying similarities that we failed to

PHOTOMICROGRAPH of thiamine (vitamin B₁), the subject of this week's Commentary (page 683). The picture is one of more than 115 kaleidoscopic images of vitamins, flavours and fragrances, sweeteners, drugs, hormones, moon rocks and superconductors presented by Michael W. Davidson in his *Magical Display: The Art of Photomicrography* (Amber Lotus, \$24.95 (pbk)).



recognize owing to surface differences, we are no less right to be cautious of false analogies that latch on to surface similarities between two disparate situations but ignore critical differences between them. There is, I believe, a crucial difference between the principles governing learning or the acquisition of knowledge and those governing evolution by natural selection. Natural selection is blind: all that matters is that, for some reason or other, certain phenotypes are more likely to reproduce themselves than others. Those phenotypes have no knowledge of what they are doing that is so successful. Indeed. Natural selection is not interested in the mechanisms or processes underlying successful adaptations: it selects only for consequences.

For Plotkin, however, behaviour that acts upon and is adapted to the world is knowledge about the world — because it is informed by constraints imposed by the world. One can see what is meant here, but still believe that an important distinction is being lost by this extension of the notion of knowledge. Thus Plotkin wants to argue that, just as when I cough to attract someone's attention, I am not simply coughing as part of normal respiratory function, so a male dog who lifts his back leg to urinate is not just urinating, he is scent marking. So he is. But the difference between my coughing and the dog's urinating is that I know what I am up to and the dog (in this instance) does not. His behaviour is 'instinctive': it is not an intentional action performed in order to achieve a certain outcome he has in mind. But when we turn to the dog's learned changes in behaviour, we are dealing with a quite different situation. Here it is abundantly clear that the reason why the dog's behaviour changes in accordance with the law of effect is precisely because he learns about the consequences of his actions. In a reasonably strict sense, he does know what he is up to. It is precisely this knowledge that is missing from Skinner's version of the law, and that allows to draw the analogy with evolution by natural selection. But the analogy is flawed.

Plotkin allows the possibility that goal-directed learning is subject to a Lamarckian rather than a Darwinian selection process, but I doubt whether this concession actually addresses the way in which the analogy fails. A rat in a Skinner box may indeed initially generate responses at random without their being directed towards a goal: in other words, the generation of variation may indeed be random and Darwinian rather than directed and Lamarckian. The critical question is how the selection process operates. Here, both Darwinian and Lamarckian processes are blind. But learning is not: we learn to perform some actions rather than others precisely because we know what the consequences of

those actions will be.

It would be quite wrong to end on such a churlish note. Plotkin has written a book that is a pleasure to read, will instruct a wide audience and will force most readers to think for themselves about the issues he addresses. He has certainly forced me to address his arguments. That I happen to disagree with him is much less important. Other readers should take the opportunity to make up their own minds. □

Nicholas Mackintosh is in the Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB, UK.

Models and molecules

Barbara Baird

Receptors: Models for Binding, Trafficking and Signalling. By Douglas A. Lauffenburger and Jennifer J. Linderman, *Oxford University Press: 1993. Pp. 365. £50, \$69.95.*

PHYSIOLOGICAL communication and responses are mediated by individual cells that sense their environment through surface receptors binding to external ligands. The central importance of binding has been revealed after many years of work by cell and molecular biologists and biochemists. We now know a great deal about the receptor and ligand molecules that combine to initiate growth and differentiation, immunological defence and a variety of other vital processes. Chemical engineers also seek an understanding of complex cellular processes in the hope of finding practical applications. They compare fairly simple mathematical descriptions and models with experiment.

Lauffenburger and Linderman are chemical engineers who are aware of the powerful synergy that can come from collaboration with cell biologists. Writing with enthusiasm but care, they go a long way towards their stated goal of bridging the gap between these two groups of workers. Even those such as biophysical chemists who already consider themselves part of the bridge will have a lot to gain from the book: it provides a broad view of biomolecular modelling from simple monovalent receptor–ligand binding in solution to complicated receptor-mediated processes resulting in cellular proliferation, adhesion and migration.

After a friendly introduction comes a meaty chapter on the fundamentals of receptor–ligand binding. This chapter will be useful to cell biologists now beginning to evaluate the binding properties of receptor–ligand systems with simple

equilibrium and kinetic measurements and data analyses. The authors develop the appropriate equations in a straightforward way, with cautious use of Scatchard and other types of popular plots. Applications are illustrated with examples from previously published work. The authors provide brief descriptions but little critical assessment of experimental methods.

Chapters 3, 5 and 6 deal with the increasingly complex receptor/ligand-mediated processes of trafficking through the cell, signal transduction and cell behavioural responses. As throughout the book, these chapters emphasize the development of descriptive and predictive mathematical models that can be tested experimentally. The focus is necessarily on the results obtained from a limited amount of successful modelling. Although only a handful of cell biologists will be interested in the details, it is worth having the broad picture of progress. In Chapter 4, the authors draw attention to the subtle physical aspects of receptor–ligand binding on cell surfaces, including probability and diffusion and multivalent binding and crosslinking. Researchers would do well to pay heed to these issues, clearly presented here, because they may well be important in the regulation of some cellular responses.

Each chapter and major section begins with a conversational overview of the biology and mathematics and provides the necessary background. Limitations in the presentation are acknowledged. The book builds on selections from a large amount of literature and gives extensive references to original sources. With the aid of this effective approach and a little effort, cell biologists will be able to learn, or at least learn to appreciate, the power of mathematical modelling. Any maths phobia is alleviated by the authors' verbal explanations of how the equations describe the processes and what a set of mathematical results really means in physical or biological terms. The list of nomenclature at the end of each chapter prevents the head from reeling with abstract symbols of forgettable meaning.

The value of this book to chemical engineers is that it exposes them to the wealth of receptor-mediated cellular processes that are increasingly amenable to mathematics. This guided glimpse of biology comes with an organized set of mathematical tools for advancing molecular understanding. Most importantly, the book provides a common language in which cell biologists and chemical engineers, and those in between, can talk to each other. This is a strong contribution to a surging interdisciplinary effort. □

Barbara Baird is in the Department of Chemistry, Baker Laboratory, Cornell University, Ithaca, New York 14853-1301, USA.

Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1

Yukiko Kurihara^{*}, Hiroki Kurihara^{*}, Hiroshi Suzuki^{*†}, Tatsuhiko Kodama^{*}, Koji Maemura^{*}, Ryoza Nagai^{*}, Hideaki Oda[‡], Tomoyuki Kuwaki[§], Wei-Hua Cao[§], Nobuo Kamada[†], Kouichi Jishage[†], Yasuyoshi Ouchi^{||}, Sadahiro Azuma[¶], Yutaka Toyoda[¶], Takatoshi Ishikawa[‡], Mamoru Kumada[§] & Yoshio Yazaki^{*#}

^{*} The Third Department of Internal Medicine, [†] Department of Pathology, [§] Department of Physiology, ^{||} Department of Geriatrics, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-Ku, Tokyo 113, Japan

[‡] Chugai Pharmaceutical Co. Ltd, Komakado, Gotenba, Shizuoka 412, Japan

[¶] Department of Reproductive and Developmental Biology, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan

The endothelin-1 (ET-1) gene was disrupted in mouse embryonic stem cells by homologous recombination to generate mice deficient in ET-1. These ET-1^{-/-} homozygous mice die of respiratory failure at birth and have morphological abnormalities of the pharyngeal-arch-derived craniofacial tissues and organs. ET-1^{+/-} heterozygous mice, which produce lower levels of ET-1 than wild-type mice, develop elevated blood pressure. These results suggest that ET-1 is essential for normal mouse development and may also play a physiological role in cardiovascular homeostasis.

ENDOTHELIN-1 (ET-1) is a 21-amino-acid peptide first reported as a potent vasoconstrictor produced by cultured porcine endothelial cells¹. Three isopeptides (ET-1, 2 and 3) constitute a gene family and share common receptors with different affinities². Among members of the family, only ET-1 is synthesized by vascular endothelial cells². In addition to the vasoconstrictive and mitogenic effects on vascular smooth muscle cells^{1,3}, ET-1 acts on endothelial cells in an autocrine manner and induces the release of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) and prostacyclin⁴. Furthermore, ET-1 has numerous effects on various tissues, including stimulation of the secretion of atrial natriuretic peptide⁵ and aldosterone⁶ and inhibition of renin release⁷. These effects of ET-1 suggest that it has a role in maintaining homeostasis of the circulatory system and the pathogenesis of cardiovascular diseases such as hypertension, vasospasm and atherosclerosis. However, there has been little consensus about the physiological and pathological importance of ET-1 in the cardiovascular system. ET-1 is also produced in many other tissues such as the central nervous system and has effects on various kinds of target cells⁸. The function of ET-1 in non-cardiovascular systems remains unclear.

In the present study, we disrupted the ET-1 gene in mouse embryonic stem (ES) cells by homologous recombination to create mice deficient in ET-1 and examined the phenotype of ET-1 deficient mice to elucidate the physiological role of ET-1. We demonstrate that the mutation in the ET-1 gene causes abnormal manifestations in fetal development and haemodynamics.

Targeted disruption of ET-1 gene in ES cells

To disrupt the ET-1 locus in mouse ES cells, we used the positive-negative selection method⁹. The targeting vector

pE10.29NEO-TK was constructed from a 7.5-kilobase (kb) fragment encompassing exons 2 to 5 of the mouse ET-1 gene (Fig. 1). Exon 2, which encodes the mature 21-amino-acid ET-1 (refs 10, 11 and our unpublished data), was disrupted by insertion of the neomycin-resistance gene (*neo^r*). The genomic fragment was flanked at the 3' end by the herpes simplex virus thymidine kinase gene (*tk*). A3.1 ES cells (derived from the 129/SvJ mouse strain)¹² were electroporated with the linearized targeting vector and clones resistant to G418 and gancyclovir were screened. Polymerase chain reaction (PCR) and Southern analysis revealed that 35 of 281 G418/gancyclovir-resistant clones were targeted for the ET-1 gene (Fig. 1d). We injected the targeted ES clone C31 into C57BL/6J mouse blastocysts and obtained two male germ-line chimaeras, which were subsequently bred with ICR or C57BL/6J females to produce mice heterozygous for ET-1 mutation.

We again screened for targeted ES cells and obtained other independent germ-line chimaeras from one of the targeted clones. ET-1^{-/-} homozygous mice derived from these germ-line chimaeras represented the same phenotype described below.

Neonatal lethality in ET-1^{-/-} homozygous mice

Mice heterozygous for the ET-1 mutation appear normal and were fertile. However, no viable homozygotes were found when 27 offspring from heterozygous crosses were genotyped at 3 weeks of age. To investigate the lethality of ET-1^{-/-} homozygotes, we observed the appearance and fate of embryos from the intercross of heterozygotes by caesarian sections at 18.5 days post-coitum (d.p.c.) and determined their genotypes. Offspring derived from 28 intercrosses of F₁ to F₅ heterozygotes derived from ICR or C57BL/6J mice were examined. Of 239 offspring delivered by caesarian sections, 60 viable ET-1^{-/-} homozygous mice with beating hearts were obtained. An example of genotyping of a litter is given in Fig. 1e. Although the heterozygous

To whom correspondence should be addressed.

and wild-type newborn mice began to breathe and turned pink within 10 min, none of the homozygotes opened their mouths to breathe, and all responded poorly to tapping and pinching stimuli. They remained blue and eventually died of anoxia within 15–30 min. Examination of their lungs showed that they had never been ventilated.

We also examined ET-1 expression by reverse transcription (RT)-PCR and enzyme immunoassay. As expected, RT-PCR revealed no expression of the ET-1 gene in homozygous embryos (Fig. 1e). No ET-1 peptide was detected in lung extracts from

homozygotes (Fig. 1f). ET-1 levels in lung extracts from heterozygotes were about 60% of those from wild-type littermates (Fig. 1f).

Morphology of ET-1^{-/-} homozygous mice

In all the 60 ET-1^{-/-} homozygous newborn mice examined, the same pattern of conspicuous craniofacial abnormalities was found. The mandible was poorly developed and its fusion in the midline was incomplete in the homozygous newborns (Fig. 2a, c). In addition, the anterior neck was thin and auricles were

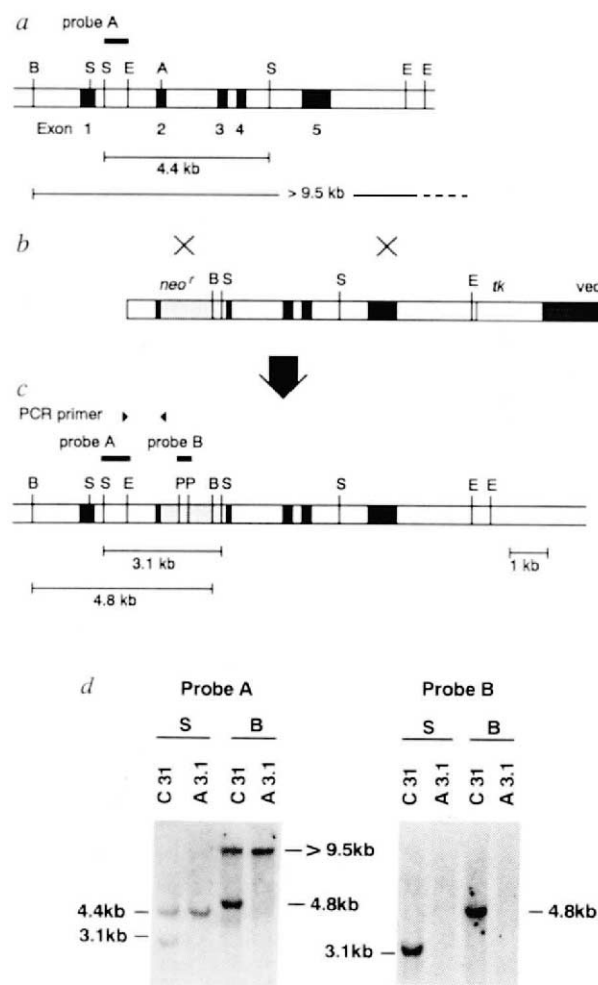
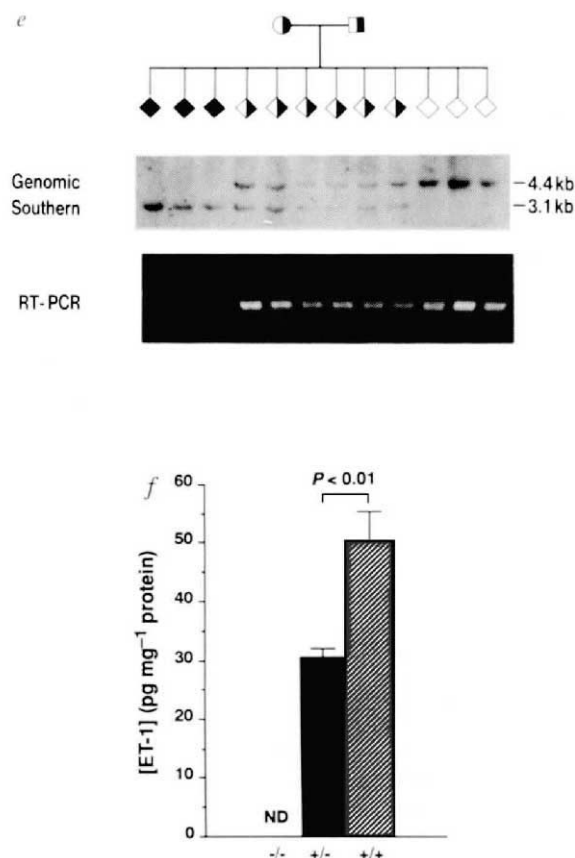


FIG. 1 Targeting of the mouse ET-1 gene. *a*, Genomic structure and restriction map of the mouse ET-1 locus. *b*, Targeting vector used to disrupt the ET-1 gene. *c*, Predicted structure of the targeted ET-1 locus. Black boxes represent exons 1 to 5. The length of diagnostic fragments, the PCR primers and the probes for Southern analysis are shown. A, *AccI*; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I. *d*, Southern blot analysis of genomic DNA from the parental ES cell line A3.1 and the targeted ES cell line C31. The 3.1-kb *Sac*I(S) fragments and the 4.8-kb *Bam*HI(B) fragments indicate the homologous recombinant allele. *e*, Southern blot analysis of *Sac*I-digested genomic DNA from a litter of 12 newborns and RT-PCR of total RNA from whole-body extract of the same littermates. Empty symbols, wild-type genotype; half-filled, heterozygous; filled, homozygous. *f*, Determination of ET-1 peptide levels in the lung from newborns. —/—, homozygotes ($n=4$); +/- heterozygotes ($n=9$); +/+, wild-type mice ($n=7$); ND, not detected.

METHODS. The mouse ET-1 genomic DNA was cloned from a BALB/c mouse genomic library in EMBL3 using synthetic oligonucleotide probes derived from the mouse ET-1 cDNA sequence⁴². The targeting vector pE10.29NEO-TK was constructed from the 7.5-kb *Eco*RI-*Eco*RI fragment containing exons 2 to 5 in pBluescriptSK+ (Stratagene). We



inserted a 1.8-kb *Xho*I-*Sac*I fragment of pGK1neo-poly(A)⁺ into the *Acc*I

site in the exon 2 using an adaptor and flanked a 1.8-kb *Xho*I-*Sac*I fragment of HSV-tk gene 3'-downstream to the ET-1 sequence. A2.1 ES cells were electroporated with pE10.29NEO-TK linearized with *Not*I and selected in medium containing G418 (300 μ g ml⁻¹) and gancyclovir (2 μ M). PCR-positive genomic DNA samples from survived ES cells were digested with *Sac*I or *Bam*HI, electrophoresed through 0.7% agarose gels, transferred to nylon membranes and hybridized to the probes at high stringency. The signals were analysed by autoradiography. Genomic DNA samples from the tails of mice were also analysed in the same manner. For RT-PCR, total RNA was prepared from whole newborn bodies using RNazol (BIOTEX) and was reverse-transcribed. PCR was done on the cDNA samples using a GeneAmp PCR kit (Perkin Elmer Cetus) and primers. Sense (5'-TGTCTTGGGAGCCGAACCTCA-3') and anti-sense (5'-GCTCGGTTGTGCGTCAACTTCTGG-3') primers were deduced from the sequence of exons 2 and 5, respectively. Twenty-eight cycles (95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min) were used to amplify 537-bp products, which were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. The lung extracts were prepared from 18.5 d.p.c. newborn littermates and ET-1 levels were measured by enzyme immunoassay^{43,44}. Statistical analysis used Student's *t*-test. Data are presented as mean $\pm$ s.e.m.

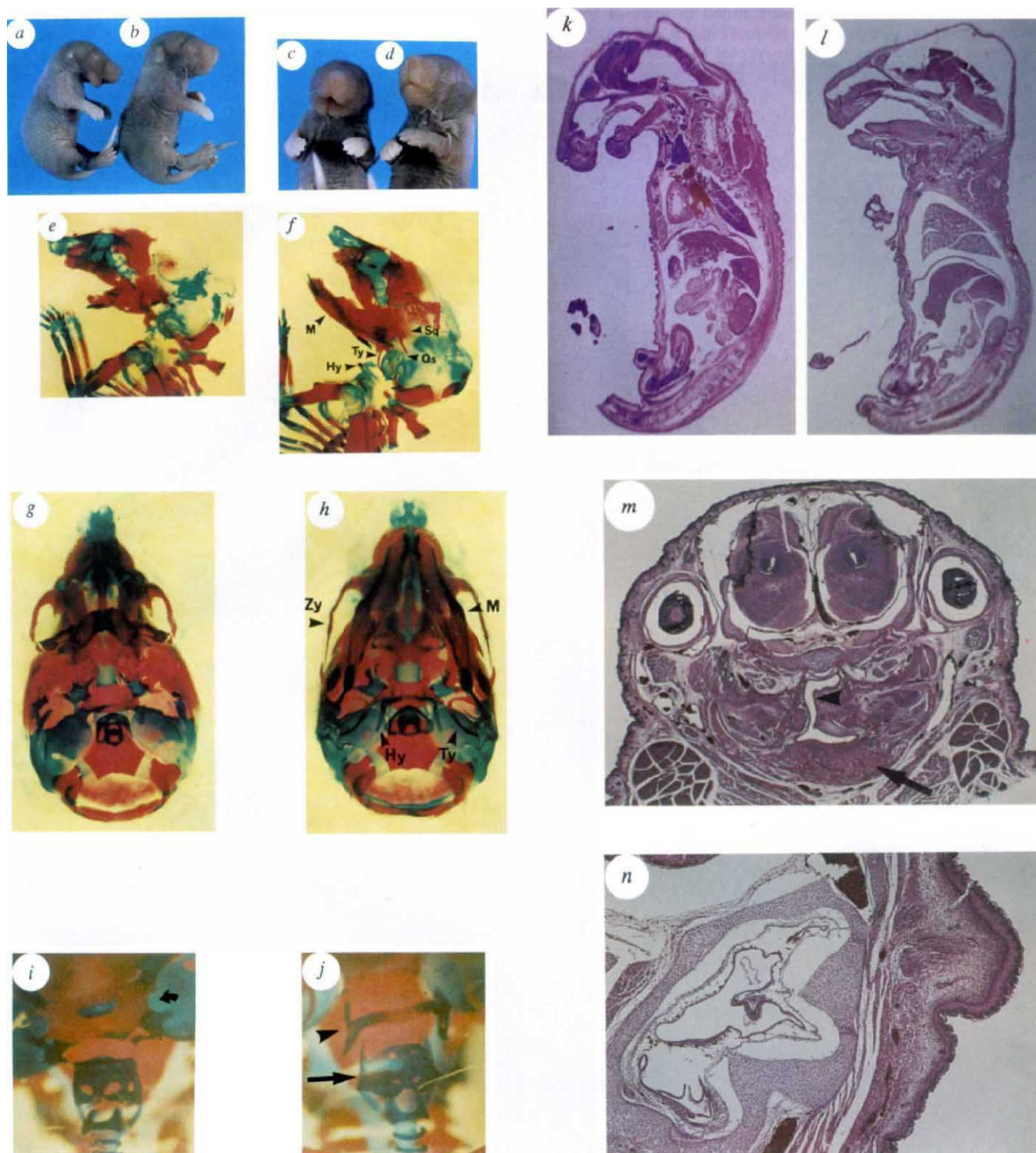


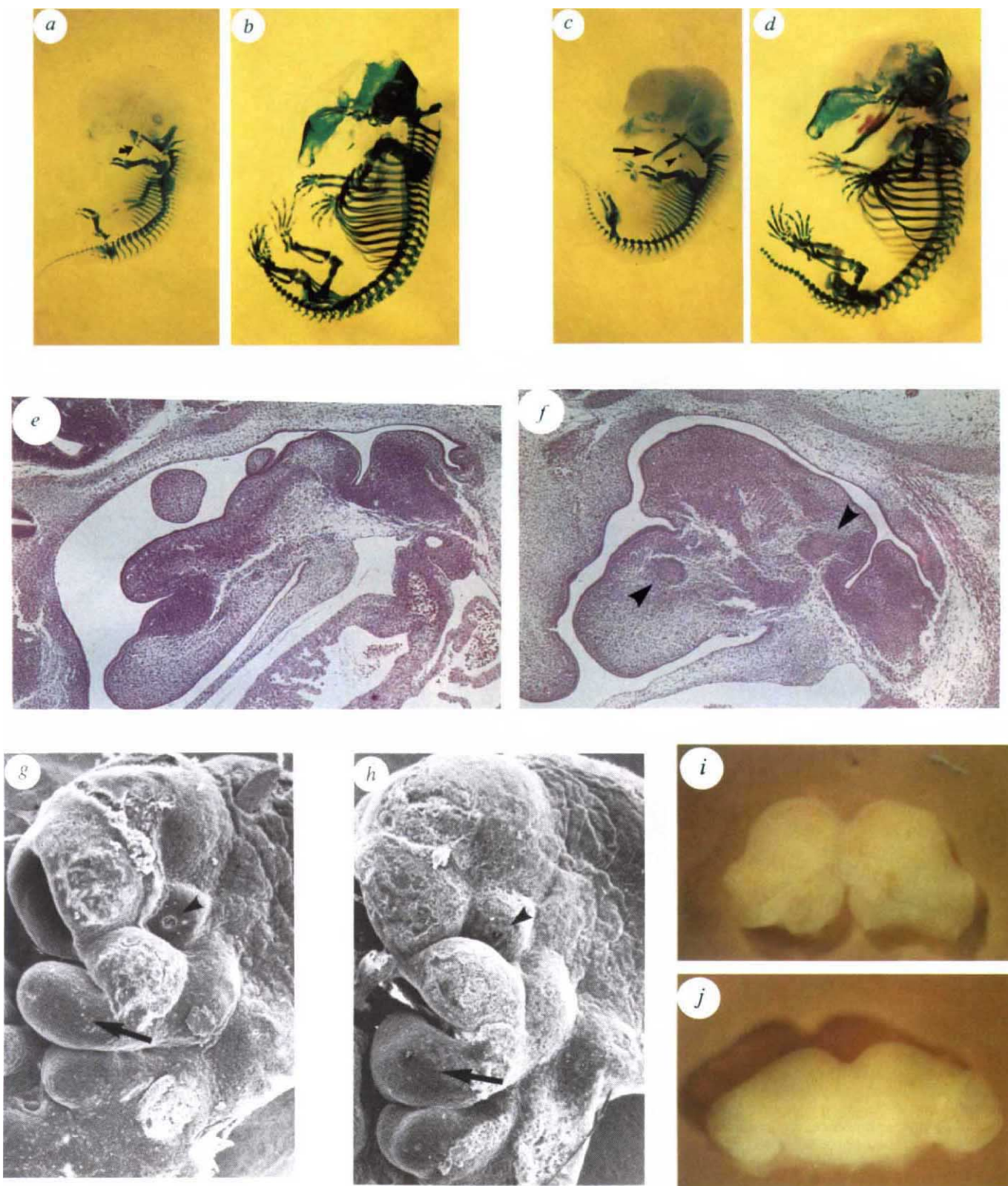
FIG. 2 Comparison of body shapes (a–d), skeletons (e–j), and histological sections (k, l, sagittal; m, n, coronal) of ET-1^{-/-} homozygous (a, c, e, g, i, k, m and n) and ET-1^{+/+} wild-type (b, d, f, h, j and l) newborn mice at 18.5 d.p.c. Homozygous mice show a short and non-fused mandible, thin anterior neck and hypoplastic auricles (a and c). For whole-mount analysis of skeletons, mice were fixed in 95% ethanol, stained with alizarin red S for bone and alcian blue for cartilage, and cleared by treatment with KOH as described⁴⁵. e–h, The homozygotes had short and deformed mandibular bones, aberrant zygomatic and temporal bones, and absent auditory ossicles and tympanic ring. Hy, hyoid; M, mandibular bone; Sq, squamosal part of temporal bone; Os, auditory ossicles; Ty, tympanic ring; Zy, zygomatic bone. i, j, Higher magnifications of the region around the hyoid (large arrow) and thyroid

cartilage (arrowhead). Deformed thyroid cartilage and no hyoid were seen in the homozygotes. Instead, an abnormal doughnut-shaped structure (small arrow) was detected. For histological analysis, newborn mice obtained from caesarian sections were fixed in 4% paraformaldehyde and embedded in paraffin. The entire mouse was serially sectioned into 8 μ m sections in sagittal and coronal planes, stained with haematoxylin–eosin, and all the sections were examined. Note that the mandibular bone and cartilage were poorly developed, whereas the dental tissue appears to be normal in the homozygous mice (k). Most of the tongue is missing (k and m, arrow). Cleft palate is also found (m, arrowhead). The outer ear is small and lacks cartilage and external auditory meatus, whereas the inner ear is normal (n).

hypoplastic. These anomalies were found in none of the 179 heterozygous or wild-type newborns (Fig. 2*b, d*). Analysis of skeletons revealed that the homozygotes had severely retarded mandibular bones, aberrant zygomatic and temporal bones, and absent auditory ossicles and tympanic ring (Fig. 2*e-h*). Thyroid cartilage was deformed and no hyoid was seen in the homozygotes (Fig. 2*i, j*). Instead, an abnormal doughnut-shaped structure, which appeared to be the result of a fusion between the

hyoid and basisphenoid bone, was detected (Fig. 2*i*) and a stereoscopic examination revealed that the trachea and oesophagus passed through the narrow hole of this abnormal structure.

Histological examination of ET-1^{-/-} homozygous newborn mice indicated that formation of the bone and cartilage is poor in the mandible (Fig. 2*k, l*). In addition, ET-1^{-/-} homozygotes lacked a large part of the tongue (Fig. 2*k-m*). The basal region of the tongue remained in many of the homozygotes, but the



muscle fibres were apparently sparse and irregular in comparison to those in the heterozygous or wild-type embryos. In the anterior neck region, the submandibular gland, muscle and connective tissue were poorly developed. A cleft palate was also found in homozygous mice (Fig. 2m). In the sections of the middle and outer ear, the auricular cartilage, external auditory meatus and most of the middle ear structure were absent, whereas the inner ear appeared to be intact (Fig. 2n).

In contrast to these anomalies in the craniofacial region, ET-1^{-/-} homozygous mice had no detectable abnormalities in any other organs such as the heart, lung, trachea, kidney and great vessels including ductus arteriosus, which are major target organs for the effect of ET-1. There were also no structural abnormalities in the central nervous system including pons, medulla oblongata and ganglia of the V, VII, VIII, IX and X cranial nerves in the homozygotes (T. Iwatsubo *et al.*, unpublished data).

To investigate the genesis of the morphological abnormalities in the homozygotes, we examined ET-1^{-/-} homozygous embryos at earlier stages. Serial staining for skeletons revealed that the development of Meckel's cartilage and hyoid was severely impaired in the homozygotes, whereas other skeletal components were normal (Fig. 3a-d). In wild-type mice, Meckel's cartilage and hyoid was apparent at 13.5 d.p.c. (Fig. 3c) and membranous ossification in the mandible occurred before 15.5 d.p.c. (Fig. 3d). In ET-1^{-/-} homozygotes, however, only a small cartilage appeared in the hypoplastic mandible at 15.5 d.p.c. (Fig. 3b). The hyoid was absent, but an abnormal doughnut-shaped cartilage was seen at 13.5 d.p.c. in ET-1^{-/-} homozygotes (Fig. 3a). Homozygous embryos at 12.5 d.p.c. has a hypoplastic mandibular arch with no precartilaginous primordium (Fig. 3e, f). Scanning electron microscope examination of embryos at 10.5 d.p.c. showed that swelling of the medial surface of the mandibular arch was poor and the second pharyngeal arch was relatively small (Fig. 3g, h). When dissected mandibular arch was examined, the lateral lingual swellings were poorly developed and the fusion was incomplete (Fig. 3i, j). These results suggest that the development of pharyngeal arches, including cartilage and tongue formation, are disturbed from the early stage of organogenesis in ET-1^{-/-} homozygotes.

The investigate further the possible involvement of ET-1 in the development of pharyngeal arches, we analysed the effect of ET-1 on the mandibular organogenesis in the organ culture sys-

tem. Mandibular arch explants from 9.5 d.p.c. wild-type embryos grown for 14 days developed tongue epithelium, cartilage, teeth and glandular tissues ($n=23$) (Fig. 4a). Explants from homozygous embryos at the same stage were characterized especially by poor development of tongue epithelium in the culture condition used ($n=10$) (Fig. 4b). When 1×10^{-8} M of ET-1 was added to culture media, the development of tongue epithelium was greatly improved ($n=4$) (Fig. 4c). Thus, ET-1 has a stimulating effect on the development of tongue epithelial tissue.

The possible role of ET-1 in ontogeny was also studied using whole-mount *in situ* hybridization. High ET-1 gene expression was detected in the pharyngeal arch region in 9.5 d.p.c. embryos (Fig. 5a), indicating a close correlation between ET-1 expression and pharyngeal arch development. Apparently, the signal was localized in the inner surface of pharyngeal arches rather than along blood vessels. The localization of the signal in the epithelium in this area was confirmed by examining frozen sections (data not shown). Lower levels of ET-1 expression were also seen as a linear pattern in the centre of embryo bodies, which may correspond to the dorsal aorta (Fig. 5a).

Elevated blood pressure in heterozygous mice

Because ET-1 has a potent pressor effect, we hypothesized that a decrease in ET-1 production may cause hypotension in ET-1 mutant mice. As ET-1^{-/-} homozygous mice were not viable after birth, we assessed the ET-1 levels and arterial blood pressure in ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice derived from the C31 targeted ES clone. In heterozygous mice, the ET-1 levels in the lung and plasma were decreased in comparison to those in wild-type mice (Fig. 6a, b). In 13 heterozygous and 9 wild-type littermates of 8 to 10 weeks old, arterial blood pressure was measured through a catheter placed in the femoral artery under conscious and unrestrained condition. Mean, systolic and diastolic blood pressures were 116 ± 2 , 133 ± 2 and 99 ± 2 mmHg, respectively, in ET-1^{+/-} heterozygous mice, and 105 ± 2 , 122 ± 2 and 88 ± 3 mmHg, respectively, in ET-1^{+/+} wild-type mice (Fig. 6c). Unexpectedly, arterial blood pressure was significantly higher in ET-1^{+/-} mice than that in ET-1^{+/+} wild-type mice ($P < 0.01$). Heart rate in ET-1^{+/-} heterozygous mice (591 ± 14 beats min^{-1}) was not different from that in wild-type mice (596 ± 26 beats min^{-1}) at the age examined (Fig. 6d).

To examine whether the elevation of blood pressure in ET-1^{+/-} heterozygous mice could be caused by an increase in vascular sensitivity to ET-1 or a decrease in EDRF/NO production, we studied the effect of ET-1 on blood pressure under conscious and unrestrained conditions and the effect of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) under anaesthetized conditions in ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice. When ET-1 (1 nmol kg^{-1}) was administered intravenously to conscious and unrestrained mice, there were no significant differences in the pressor effect between ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice (Fig. 6e). Initial transient depressor responses, which are characteristic for ET-1 in other species, were too small to evaluate in both groups. Under anaesthetized conditions, the blood pressure of ET-1^{+/-} heterozygous mice was also significantly higher than that of ET-1^{+/+} wild-type mice. The pressor response to 250 $\mu\text{mol kg}^{-1}$ L-NMMA did not differ between ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice (Fig. 6f).

Discussion

We disrupted the ET-1 gene in mouse ES cells by gene targeting and established mice bearing the mutant ET-1⁻ allele. The mutation was introduced by inserting *neo'* into exon 2, which encodes mature ET-1 peptide, so that no protein containing active ET-1 peptide sequence is translated. This is confirmed by the fact that no authentic ET-1 messenger RNA or peptide were detected in ET-1^{-/-} homozygote tissue. The resulting phenotype is characterized by lethality and malformations of craniofacial tissues in ET-1^{-/-} homozygotes and elevated blood pressure in

◀ FIG. 3 Comparison of skeletons of 13.5 (a and c) and 15.5 (b and d) d.p.c. embryos, sagittal sections of 12.5 d.p.c. embryos (e and f), and scanning electron micrographs (g and h) and morphology of dissected mandibles (i and j) of 10.5 d.p.c. embryos. a, b, e, g, and i are ET-1^{-/-} homozygous and c, d, f, h, and j ET-1^{+/+} wild-type mice. In wild-type mice, Meckel's cartilage (c, large arrow) and hyoid (c, arrowhead) was apparent at 13.5 d.p.c. and membranous bones in the mandible appeared at 15.5 d.p.c. (d). In homozygotes, only a small cartilage appeared in the hypoplastic mandible at 15.5 d.p.c. (b). The hyoid was absent, but an abnormal doughnut-shaped cartilage (a, small arrow) was already seen at 13.5 d.p.c. in homozygotes. In histological examination, an ill-developed first pharyngeal arch containing no precartilaginous primordium is observed in 12.5 d.p.c. homozygotes (e), whereas precartilaginous primordium is seen in wild-type littermates (f, arrowhead). For scanning electron microscopic examination, embryos were washed in PBS with 0.2% glutaraldehyde and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium cacodylate (pH 7.6). After washing in PBS, the samples were sequentially dehydrated in graded ethanols, critical point dried, coated with gold in a sputter coater, and viewed in a Hitachi S-450 LB scanning electron microscope at $\times 75$ magnification. An arrow indicates the mandibular component of the first pharyngeal arch; arrowhead, lens pit (g and h). When mandibular arches were dissected from 10.5 d.p.c. embryos and were examined using a stereoscope, the lateral lingual swellings were poorly developed and the fusion was incomplete (i and j).

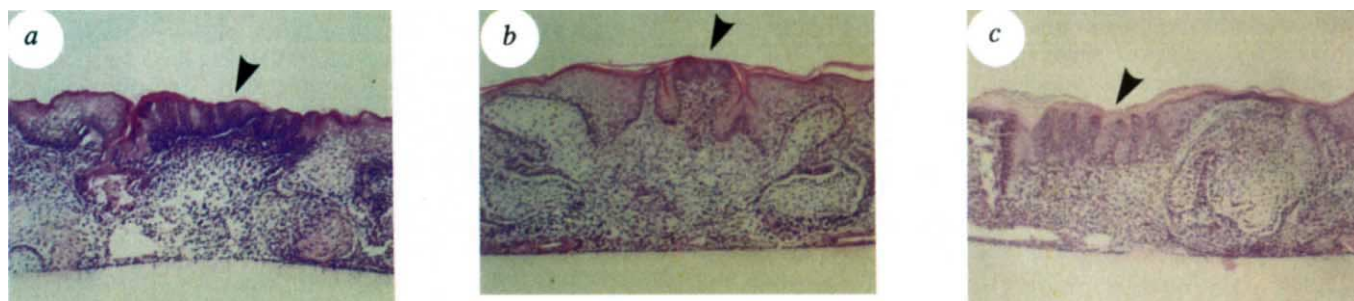


FIG. 4 *In vitro* organ culture of the mandibular arches of 9.5 d.p.c. embryos. The mandibular arches of ET-1^{+/+} wild-type mice (a) and of ET-1^{-/-} homozygous mice (b and c) were cultured in the absence (a and b) or presence (c) of 1×10^{-8} M of ET-1. All the light micrographs demonstrate the section where tongue epithelium is most prominent in each specimen.

METHODS. *In vitro* organ culture has been described⁴⁶. Mandibular arches at 9.5 d.p.c. were dissected and placed on 0.8 μ m pore size

Millipore filter paper. Each filter with mandibular arches was supported by a steel mesh grid and cultured in BGJb medium (Gibco) supplemented with 10% fetal calf serum and 0.1 mg ml⁻¹ ascorbic acid. After 14 days, cultured mandibular arches were fixed in 4% paraformaldehyde and embedded in paraffin. Serial 6- μ m sections were cut and stained with haematoxylin and eosin. Arrowheads indicate tongue epithelium. Genotypes were determined by Southern analysis of genomic DNA samples prepared from the rest of the embryos.

ET-1^{+/-} heterozygotes. These results indicate the involvement of ET-1 in normal ontogeny and suggests a role for ET-1 in haemodynamic regulation.

The cause of death in ET-1^{-/-} homozygotes is apparently anoxia due to respiratory failure. The absence of obvious abnormality in the heart and great vessels indicates that the respiratory failure is not secondary to heart failure. Rather, mechanical obstruction of the abnormal doughnut-shaped structure to the upper airway and inability to open the mouth because of poor musculature in the mandibular region may contribute to the lethality. In addition, difficulty in breathing in response to physical and noxious stimuli suggest that abnormality in central respiratory control or respiratory muscle may also be involved in the cause of lethality. Previous reports have shown that the expression of ET-1 and binding sites for ET-1 are detected in the nervous system associated with respiratory control^{13,14} and that ET-1 has both stimulatory and inhibitory effects on central respiratory control in a region-specific manner¹⁵⁻¹⁸. In our preliminary experiments, we observed the neural control of respiration is disturbed in ET-1^{+/-} heterozygous mice (T. Kuwaki *et al.*, manuscript in preparation). Although further investigation is necessary to clarify the precise mechanism of the lethality, these results suggest that ET-1 may be important in the neural regulation of the respiratory system.

The most outstanding finding in ET-1^{-/-} homozygous mice is the finding of craniofacial anomalies affecting the mandible, zygomatic and temporal bones, tympanic ring, hyoid, thyroid

cartilage, tongue, soft tissue in the anterior neck, palate and outer and middle ears. All of these organs are developed from the pharyngeal arches whose origin is mainly neural crest-derived ectomesenchymal cells¹⁹. Migration of neural crest cells into the region of pharyngeal arches completes before 10 d.p.c. and their differentiation starts thereafter²⁰. Examination of embryos at serial developmental stages suggests that disturbance in the development of pharyngeal arches already starts around this early stage and their differentiation to Meckel's cartilage, tongue primordium and other specific tissues is impaired in ET-1^{-/-} homozygous mice. It is quite unlikely that these abnormalities are due to an incidental mutation other than at the ET-1 locus because the linkage between the genotype and phenotype are complete in 239 mice, and two independent ES clones that have the ET-1 mutation gave rise to the same phenotype in ET-1^{-/-} homozygotes. Furthermore, ET-1 has a stimulatory effect on tongue development in organ culture and high ET-1 gene expression is detected in the pharyngeal arches at the early stage of embryonic organogenesis by *in situ* hybridization. These data strongly support the involvement of ET-1 in pharyngeal arch development.

ET-1 expression in the pharyngeal arches seemed to be localized in epithelial cells. It is well known that epithelial-mesenchymal interactions are pivotal events in organ development in the pharyngeal region as well as in many other regions^{21,22}. Although several growth factors, for example members of the transforming growth factor- β family, are considered to be involved in epithel-



FIG. 5 Whole-mount *in situ* hybridization of 9.5 d.p.c. mouse embryos with the ET-1 antisense and sense probes. a, Probed with the ET-1 antisense probe. b, Probed with the control sense probe.

METHODS. A 1.0-kb fragment of the mouse ET-1 cDNA was subcloned in pBluescriptSK+ and non-radioactive antisense and sense RNA probes were synthesized with digoxigenin-11-UTP (Boehringer). Whole-mount fixation and *in situ* hybridization were done according to the procedure described by Wilkinson⁴⁷. Hybridized embryos were washed at high stringency, incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody and stained with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

ial-mesenchymal interactions^{22,23}, little is known about their precise mechanism. The present data strongly suggest that ET-1 may participate in the epithelial-mesenchymal interactions to promote pharyngeal arch development. Thus, our present results are indicative of a novel physiological role of ET-1 in ontogeny in mammals.

The phenotypic manifestation of ET-1^{-/-} homozygous mice is reminiscent of mice mutant for homeotic genes such as *Hox-1.5* (ref. 24) or *Hox-1.6* (refs 25, 26), or retinoic acid-induced embryopathy^{27,28}. In humans, the ET-1 gene is located on chromosome 6 (ref. 10), on which no clusters of the homeotic gene family are located²⁹. Considering the gross similarity of chromosomal linkage between man and mice, it is unlikely that coincidental mutation of any homeotic gene contributed to the morphological abnormality in ET-1^{-/-} homozygous mice. Note that the phenotype of ET-1^{-/-} homozygous mice is quite similar to the human congenital disease known as first pharyngeal arch syndrome, including Pierre-Robin syndrome and Treacher-Collins syndrome³⁰. This syndrome is characterized by morphological abnormality of the first pharyngeal-arch-derived tissues

such as the mandible, ear, palate and eye, and is thought to be due to abnormality in the development of a specific neural crest cell lineage³¹. At present, the causative gene(s) of any subtype of this syndrome have not been identified. ET-1^{-/-} homozygous mice may be a useful tool to investigate the development of the pharyngeal arch and to clarify the pathogenesis of the first pharyngeal arch syndrome.

Mild but significant elevation of blood pressure in ET-1^{+/-} heterozygotes is also an unexpected result. The blood pressure elevation in ET-1^{+/-} heterozygotes was observed under not only conscious and unrestrained but also anaesthetized conditions, indicating that the blood pressure elevation is not due to emotional stress. A bolus injection of ET-1 induces transient reduction and long-lasting elevation in blood pressure^{1,2}. Chronic infusion of ET-1 causes sustained hypertension in conscious rats^{32,33}. In addition, a case of ET-1-secreting haemangioendothelioma accompanied by hypertension was reported³⁴. In this case, blood pressure was significantly reduced after resection of the tumour. These previous data suggest that a sustained increase in plasma ET-1 levels is positively correlated with blood

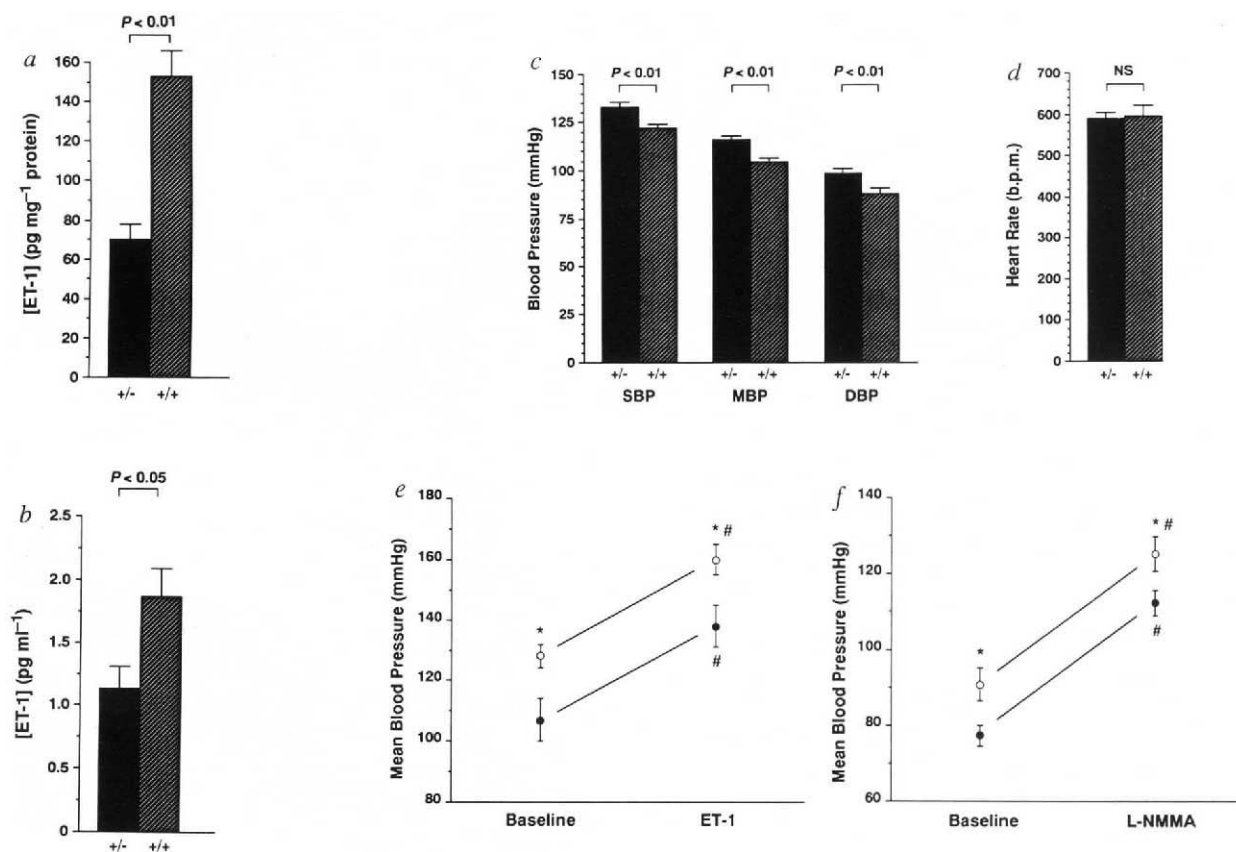


FIG. 6 Comparison of ET-1 levels in the lung (a) and plasma (b), basal arterial blood pressure (c) and heart rate (d), and the pressor effects of ET-1 (e) and L-NMMA (f) between heterozygous and wild-type mice. Eight-to-ten-week-old male littermates were used for all the experiments. a and b, ET-1 levels were determined as described^{43,44}. +/-, Heterozygotes (lung, $n=9$; plasma, $n=5$); +/+, wild-type mice (lung, $n=8$; plasma, $n=5$). c and d, Heterozygous ($n=13$) and wild-type ($n=9$) littermates were cannulated with polyethylene tubing into the femoral artery under halothane anaesthesia. On the following day, pulsatile blood pressure was measured under conscious and unrestrained condition in a quiet environment after at least 30 min of acclimatization period during 13:00–17:00. Blood pressures were determined every 2.5 s by a peak detector (AP-611G, Nihon-Kohden), stored into a tape recorder together with heart rate which was computed by a tachometer (AT-601G, Nihon-Kohden) and fed into a computer (PC9801RX, NEC) to calculate the mean value of each variable over a 2 h segment com-

posed of 2,880 sample points. +/-, Heterozygotes; +/+, wild-type mice; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure. e, Heterozygous ($n=5$) and wild-type ($n=6$) littermates were subjected to blood pressure measurement under conscious and unrestrained condition. Increases in mean blood pressure induced by an intravenous administration of 1 nmol kg⁻¹ of ET-1 were not different between ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice (32 ± 3 versus 32 ± 3 mmHg, $P=0.99$). f, Heterozygous and wild-type littermates ($n=9$, each) were subjected to experiments under anaesthesia with urethane (1.5 g kg⁻¹, i.p.). Increases in mean blood pressure induced by an administration of 250 μ mol kg⁻¹ L-NMMA were not different between ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice (35 ± 3 versus 35 ± 4 mmHg, $P=0.92$). O, Heterozygous mice; ●, wild-type mice. * $P<0.05$ versus wild-type mice; # $P<0.001$ versus baseline. Statistical comparison used Student's *t*-test. Data are presented as mean $\pm$ s.e.m.

pressure. However, the present study reveals that mice in which ET-1 production is genetically reduced develop paradoxically elevated blood pressure. The blood pressure elevation cannot be ascribed to hypersensitivity to ET-1, because the response of blood pressure to exogenous ET-1 was not different between ET-1^{+/-} heterozygous and ET-1^{+/+} wild-type mice.

Several points should be noted in order to interpret the paradoxical elevation of blood pressure in ET-1^{+/-} heterozygous mice. First, the production of ET-1 is decreased throughout the ontogeny in ET-1^{+/-} heterozygotes. Changes in ET-1 production at developmental stages may affect normal integration of the cardiovascular regulatory system. Second, the physiological activity of ET-1 may not be mimicked by an exogenous injection of ET-1 into circulating blood, because ET-1-producing cells (such as vascular endothelial cells and neuronal cells) and the target cells are identical or so close (such as vascular smooth muscle cells) that ET-1 seems to act mainly as a paracrine/autocrine factor and/or a neurotransmitter rather than as a circulating hormone³⁵. The fact that neutralizing antibodies to ET-1 do not affect blood pressure in rats also indicates that there is little contribution of plasma ET-1 on the control of blood pressure³⁶. Third, several reports have demonstrated that ET-1 not only has a pressor effect but also a depressor effect through stimulation of EDRF/NO production by endothelial cells⁴ and through an effect on the central nervous system³⁷. It should be considered whether the depressor effect of ET-1 can explain the

blood pressure elevation in ET-1^{+/-} heterozygous mice. In ET-1^{+/-} heterozygous mice, however, response to L-NMMA was not different from that in ET-1^{+/+} wild-type mice. This result suggests that reduction in EDRF/NO production may not explain the blood pressure elevation in ET-1^{+/-} heterozygotes.

In our preliminary experiments, reduction in arterial *pO*₂ associated with disturbance in neural cardiopulmonary control is detected in ET-1^{+/-} heterozygous mice (T. Kuwaki *et al.*, manuscript in preparation). This respiratory abnormality may induce blood pressure elevation. Previous reports have suggested that ET-1 serves as a neurotransmitter or neuromodulator in the central cardiorespiratory control^{15,16,37,38}. In fact, intracisternally administered ET-1 is known to alter discharges of vasomotor neurons in the rostral ventrolateral medulla, a presumed site of the vasomotor centre³⁹, and thereby affects tonic sympathetic discharges⁴⁰. A recent report showed that the ET-1 level in the cerebrospinal fluid was diminished to about a quarter of the control by the arterial baroreceptor reflex elicited by sustained increase in blood pressure⁴¹. Therefore the blood pressure elevation in ET-1^{+/-} heterozygotes may be caused by changes in the central cardiorespiratory control. Although further investigation is required for the clarification of the mechanism of high blood pressure in ET-1^{+/-} heterozygous mice, the present results suggest that ET-1 may play a role in the regulation of blood pressure as a depressor rather than as a pressor in a physiological state in mice. □

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Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen

Theodore S. Jardetzky, Jerry H. Brown^{*†}, Joan C. Gorga[‡],
Lawrence J. Stern^{*}, Robert G. Urban, Young-in Chi[§],
Cynthia Stauffacher[§], Jack L. Strominger & Don C. Wiley^{*||}

Department of Biochemistry and Molecular Biology, ^{*} Howard Hughes Medical Institute, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA

[†] Rosenstiel Research Center, Brandeis University, 415 South Street, Waltham, Massachusetts 02154, USA

[‡] Department of Pediatrics, Children's Hospital of Pittsburgh, 6130 Rangos Research Building, 3705 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA

[§] Department of Biology, Purdue University, West Lafayette, Indiana 47907, USA

The structure of a bacterial superantigen, *Staphylococcus aureus* enterotoxin B, bound to a human class II histocompatibility complex molecule (HLA-DR1) has been determined by X-ray crystallography. The superantigen binds as an intact protein outside the conventional peptide antigen-binding site of the class II major histocompatibility complex (MHC) molecule. No large conformational changes occur upon complex formation in either the DR1 or the enterotoxin B molecules. The structure of the complex helps explain how different class II molecules and superantigens associate and suggests a model for ternary complex formation with the T-cell antigen receptor (TCR), in which unconventional TCR-MHC contacts are possible.

SUPERANTIGENS comprise a class of disease-associated, immunostimulatory molecules that bind class II MHC molecules and stimulate large numbers of T cells^{1,2}. Members of the superantigen family include toxins from *S. aureus* and other bacteria³, as well as viral superantigens from mouse mammary tumour virus (MMTV)⁴. The *S. aureus* toxins are associated with food poisoning and toxic-shock syndrome, and the MMTV superantigen plays a critical role in viral transmission. The toxicity of bacterial superantigens is thought to be mediated by their potent T-cell-stimulating activities⁵, leading to lymphokine release⁶, respiratory distress and shock. Superantigens have also been implicated in rabies, rheumatoid arthritis, and mouse and human AIDS⁷.

The mechanism by which superantigens stimulate T cells differs from that of normal antigens. Conventional T-cell antigens are short proteolytic peptides from foreign proteins, bound in the peptide-binding groove of class I or class II MHC molecules⁸. The structures of both class I and class II MHC molecules⁹⁻¹¹ demonstrate that these bound peptides become an integral part of the MHC protein surface, which is displayed by antigen-presenting cells to specific T cells, generating an immune response. In contrast, bacterial superantigen activity is abolished by proteolysis and it is the intact superantigen protein that interacts with class II MHC molecules outside the peptide-binding groove^{12,13} in order to stimulate T cells.

The interaction of conventional peptide antigens and superantigens with the T-cell antigen receptor (TCR) also differs. TCR molecules are structurally related to antibody molecules, with hypervariable regions forming a combining site for a specific peptide-MHC combination¹². Superantigens bypass this specificity-determining region of the TCR, and interact with a surface of the TCR predicted to lie outside the antigen-combining site on the variable β -chain ($V\beta$) domain¹⁴⁻¹⁸. This ability

of superantigens to interact with both MHC and TCR molecules outside their normal antigen-specific sites leads to the stimulation of many more T cells than observed with normal peptide-antigens.

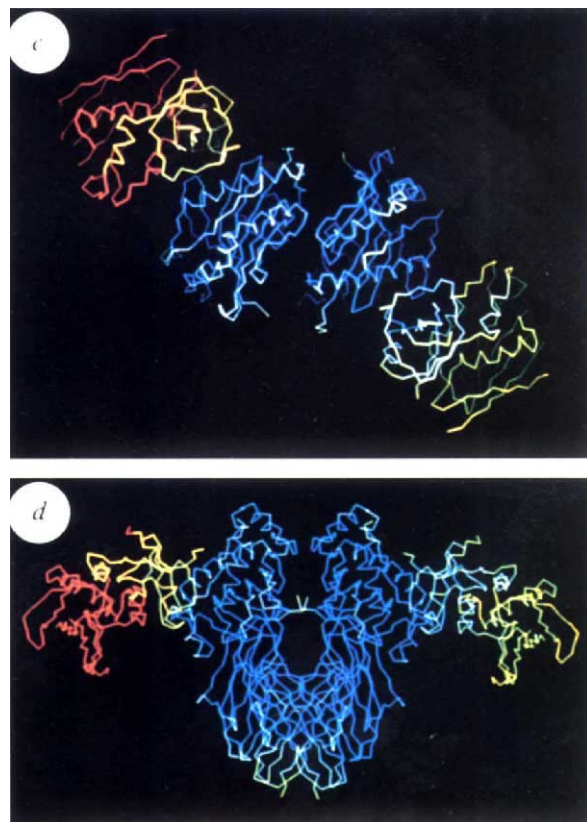
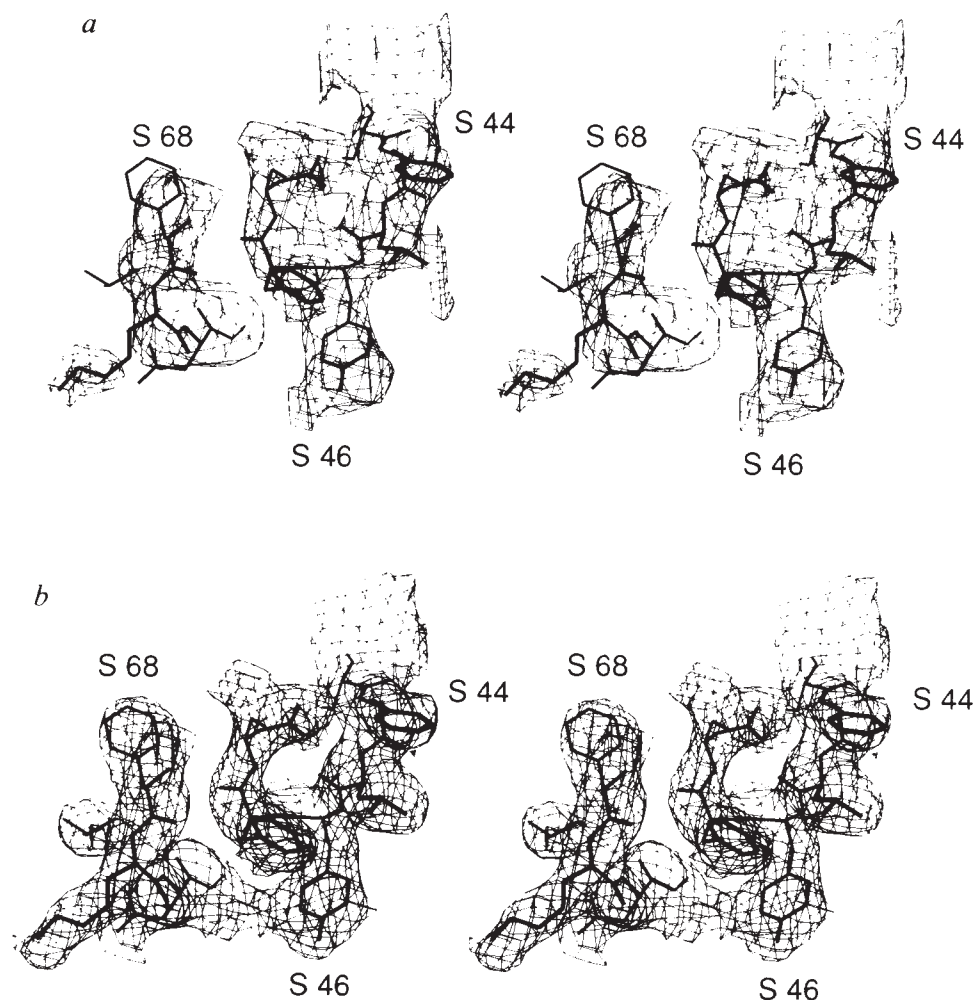
To understand better the molecular basis of the pathological effects of superantigens, we have determined the structure of a bacterial superantigen, *S. aureus* enterotoxin B (SEB), bound to a human class II MHC molecule, HLA-DR1, by X-ray crystallography. SEB binds outside the MHC peptide-binding site, with the N-terminal domain of SEB interacting with the DR1 $\alpha 1$ domain. No large conformational changes are observed in either the class II molecule¹¹ or the SEB molecule¹⁹ upon complex formation. SEB residues that affect the interaction with T-cell receptors are positioned to the side and above the DR1 peptide-binding site of the class II molecule, suggesting a model for the interaction of superantigen-MHC complexes with TCR, where normal TCR-MHC interactions are blocked.

Structure determination

The structure of the DR1-SEB complex was determined using the data sets listed in Table 1, from two different DR1-SEB crystal forms. Initial electron density maps were generated using SIR/anomalous phases for crystal form I and improved by averaging with maps derived from HLA-DR1 crystals as described¹¹. The initial model of the DR1 molecule was improved by cycles of building and refinement and a partial model for the SEB was built. A low-resolution data set of crystal form II (Table 1) was solved by molecular replacement with the partial model of the complex, and iterative non-crystallographic four-fold symmetry averaging was used (Fig. 1a) to obtain a polyaniline trace for 190 residues of the SEB molecule at low resolution. This model was used to calculate higher-resolution electron-density omit maps, followed by iterative non-crystallographic two-fold symmetry averaging, with data from crystal form I, and improved by further cycles of building and refinement (Fig. 1b).

|| To whom correspondence should be addressed.

FIG. 1 Electron density maps and temperature factors for the DR1-SEB complex. SEB electron density maps at the DR1-SEB Interface. *a*, 15.0–4.3 Å $|2F_o - F_{calc}|$ map generated by 4-fold iterative averaging between crystal form I and form II. *b*, A current 2-fold averaged $|2F_o - F_{calc}|$ omit map at 2.7 Å resolution, calculated using current model phases and data from crystal form I, omitting the atoms shown. Both maps are contoured at 1.0σ with a cover radius of 1.5 Å around the atoms shown. *c* and *d*, Top and side views (respectively) of the DR1-SEB complex showing the radial increase in temperature factor. Two DR1-SEB complexes are found in the asymmetric unit as shown. The current model is coloured by temperature factor (atomic *B*-factor), with blue representing *C α* *B*-factor values less than 25 Å² and red representing *B*-factor values greater than 100 Å² (see Table 1 for average SEB *B*-factors). Note the increase in *B*-factor as a function of distance from the DR1-SEB interface, and the correspondence with loops in the DR1 structure. The high-temperature factors may be due to SEB disorder within the crystal lattice. One SEB makes no contacts with other symmetry-related molecules in the lattice, whereas the other SEB molecule has only few crystal contacts in regions of the C-terminal domain that may not be accurately modelled. The DR1 molecules form crystal contacts in all lattice directions and may provide the predominant stabilization of the crystal lattice. The high-temperature factors may therefore also reflect a lower SEB occupancy within the lattice. Refinement of SEB occupancy before *B* refinement typically provides an improvement in *R_{free}* of 0.3–0.5%. Further experiments are necessary to resolve these possibilities.



The structure of the complex shows a gradient of disorder of the SEB molecules, extending radially out from the DR1-SEB interface (Fig. 1*c*, *d*). This has two consequences. First, five surface loops of the SEB molecules show no interpretable electron density, presumably because of the higher basal level of SEB disorder. Second, the SEB C-terminal domain shows generally weaker electron density, with stunted or absent side-chain density. The N-terminal domain of the SEB molecule, which forms most of the contacts to DR1 (Fig. 1*b*; and shown in yellow in Fig. 2*a*) is, however, the best ordered region of the SEB structure. The disorder evident in the structure does not affect either our major conclusions as to how SEB binds to class II MHC molecules or the implications for TCR interactions.

Overview of DR1-SEB complex formation

Figure 2 shows top and stereo views of the DR1-SEB complex. SEB only contacts residues of the $\alpha 1$ domain of DR1, interacting with amino acids from the first and third turns of the β -sheet and from the N-terminal region of the α -helix. These residues form a deep, concave surface to one side of the peptide-binding site of DR1 (Fig. 3*c*), in agreement with mutational studies mapping the MHC-SEB interaction¹³. The potential influence of α -chain polymorphisms in the binding of SEB to other human and mouse class II molecules is discussed later. SEB does not interact

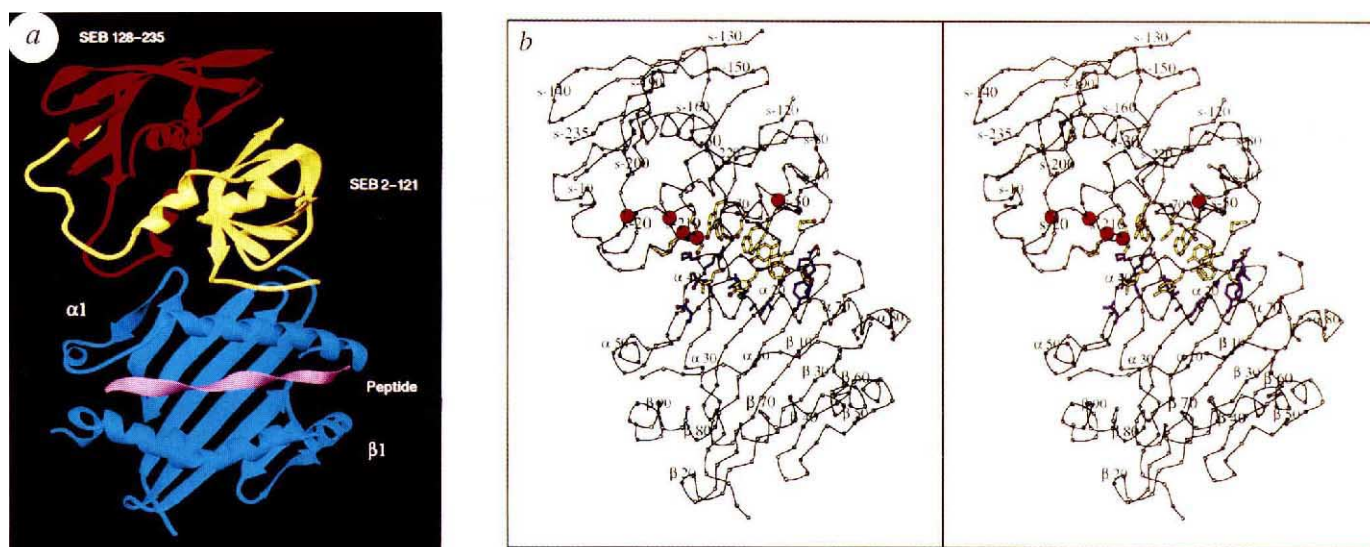


FIG. 2 Overview of the complex between HLA-DR1 and SEB. *a*, Top view, HLA-DR1 $\alpha 1$ and $\beta 1$ domains are shown in blue; $\alpha 2$ and $\beta 2$ domains are not shown. N-terminal residues of SEB 2–121 are in yellow, and C-terminal residues 127–235 of SEB are in red; the peptide is shown in pink. The peptide conformation is based on fitting a polyalanine chain into the observed electron density corresponding to a mixture of self-peptides bound to the HLA-DR1 molecule. *b*, $C\alpha$ trace of the complex (DR1 $\alpha 2$ and $\beta 2$ not shown), showing all side chains involved in the DR1–SEB interface. SEB interface residues are in yellow; DR1 interface residues are in dark blue. Red spheres mark the SEB and SEA residues implicated in TCR interactions^{2,24,41–43}.

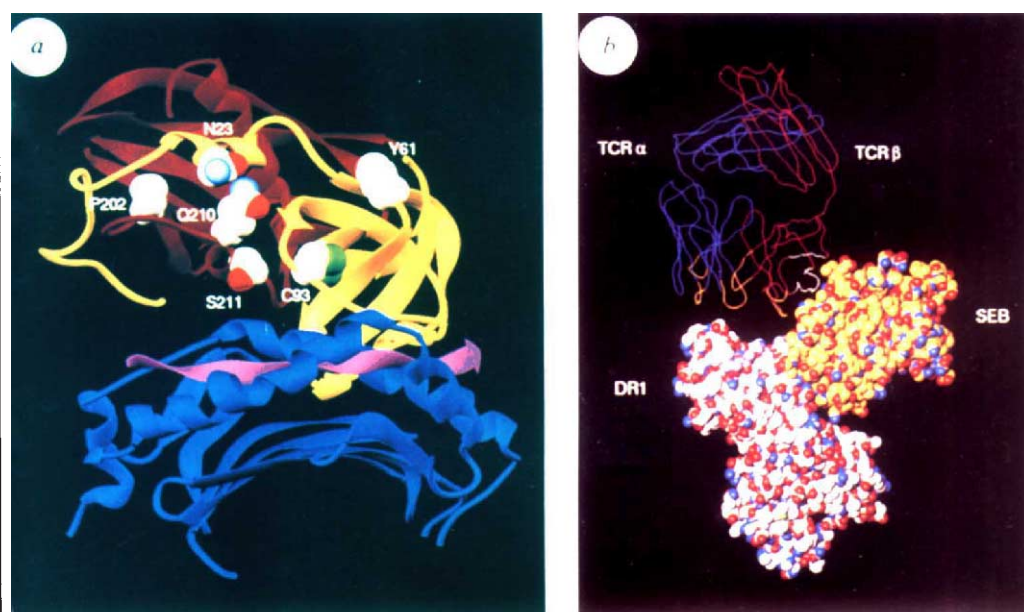
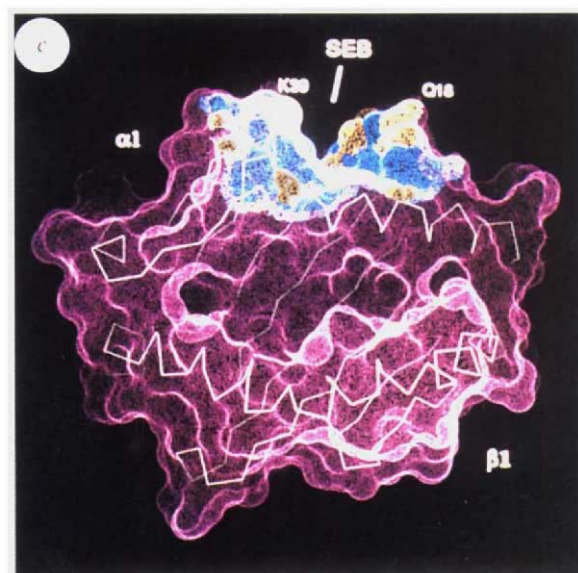


FIG. 3 Location of superantigen residues involved in TCR interactions and a model of ternary complex formation between DR1, SEB and TCR. *a*, Residues in SEB (N23, Y61, C93–C113; N60 not shown) and SEA (in SEA: G200, S206, N207; in SEB P202, Q210, S211) that have been implicated in TCR interactions by mutagenesis^{2,24,41–43}. DR1 $\alpha 1$ and $\beta 1$ domains are blue (DR1 $\alpha 2$ and $\beta 2$ domains are not shown), SEB is yellow (N-terminal residues) and red (C-terminal residues), and peptide is pink. CPK representation is white for carbon atoms, red for oxygen, blue for nitrogen, and green for sulphur. *b*, Hypothetical model of ternary complex formation of DR1 and SEB with TCR. An immunoglobulin Fab fragment model of the TCR α -chain in blue, β -chain in red. Hypervariable regions of TCR (CDR1, CDR2 and CDR3) are shown in yellow, and the HV4 loop and B strand of the $V\beta$ domain are shown in white. DR1 carbon atoms are white, SEB carbon atoms are yellow, peptide carbon atoms are magenta. The view is looking down the DR1 peptide-binding site, with the DR1 β -chain to the left and the α -chain to the right. The TCR is positioned so that the SEB residues shown in *a* can interact with the white HV4 loop. *c*, Top view of the class II molecule, showing the region of the $\alpha 1$ domain buried by complex formation with SEB. Surface of the DR1 molecule shown in magenta outside the SEB interaction area. Blue, hydrophobic surface buried by SEB; yellow, polar surface buried by SEB.



with class I MHC molecules and this region differs in class I and class II MHC structures¹¹. Good peptide density is observed in the peptide-binding site, corresponding to a mixture of self-peptides, and has been modelled as polyalanine (Fig. 2). SEB does not interact directly with the peptide and the peptide conformation is very similar to that observed for a single peptide-DR1 complex²⁰.

Residues from the SEB molecule that interact with DR1 derive predominantly from the smaller N-terminal β -barrel domain of the SEB molecule, although three residues from the C-terminal helix 5 (residues 210–217) also contact the DR1 molecule. Helix 5 is actually more closely associated with the N-terminal domain than with the C-terminal domain (Fig. 2b). The SEB residues most central to the interface lie in a turn between strands 1

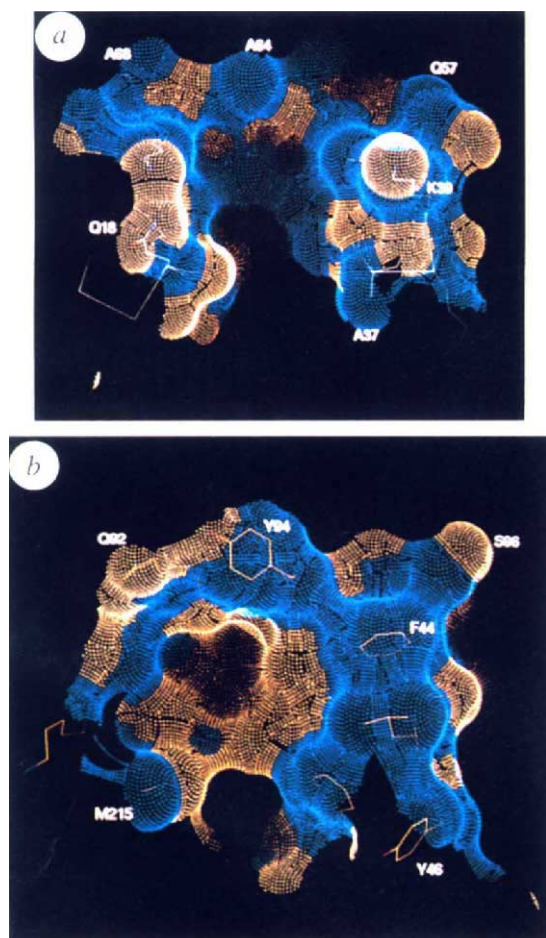
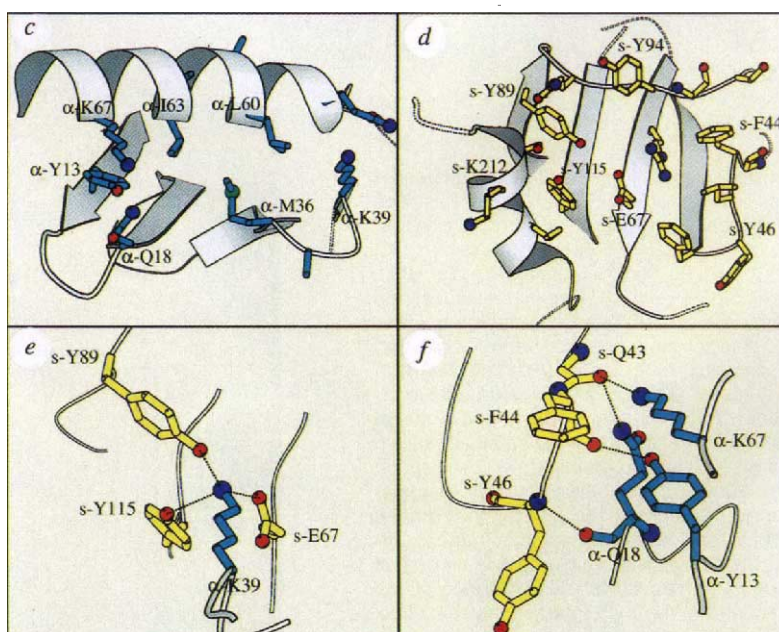


FIG. 4 The DR1-SEB binding interface. *a*, Surface view of the DR1 residues involved in binding to SEB. Yellow, polar atoms at surface; blue, hydrophobic atoms at surface. *b*, Surface view of the SEB residues involved in binding, same colour scheme as in *a*. *c*, DR1 residues involved complex formation, side view. DR1 residues are shown with blue bonds. Red, green and dark blue spheres represent oxygen, sulphur and nitrogen atoms, respectively. *d*, SEB residues involved in complex formation. SEB residues are shown with yellow bonds and atoms coloured as in *c*. *e*, Salt bridge formed between DR1 α -chain residue lysine 39 and SEB glutamic acid 67, with SEB tyrosines 89 and 115 forming hydrogen bonds. *f*, Potential hydrogen bonds formed between residues of the DR1 molecule and the main chain of residues 43–46 of the SEB molecule. Surfaces generated with MS²³ using a probe radius of 1.4 Å, and displayed in O⁶⁴; *c–f* generated with Molscript⁶⁵. Surfaces areas quoted in the text were calculated with the default atomic radii used by the program Access²² and a probe radius of 1.4 Å.



(residues 33–39) and 2 (residues 48–52) and along strand 3 (residues 63–68). In addition, a stretch of residues in the SEB disulphide loop (residues 92–96) runs above the binding interface parallel to the DR1 α 1 α -helix. The C-terminal domain of the SEB molecule is oriented up and away from the class II molecule, consistent with the observation that N-terminal constructs containing residues 1–138 of the SEB molecule bind class II MHC molecules and retain partial activity²¹.

Description of the interface

Figure 2b shows a stereo view of the DR1 and SEB residues involved in complex formation. The interface is comparable in size to antigen–antibody interfaces, burying 780 Å² and 760 Å² of the DR1 and SEB solvent-accessible²² surfaces respectively. Twenty-one residues of the DR1 molecule and nineteen residues from the SEB molecule are involved in complex formation.

A topological view of the binding interface shows a dramatic division of the complementary surfaces of the DR1 and SEB molecules. Figure 4a, b shows the molecular surfaces²³ of the DR1 and SEB molecules respectively, which are buried upon formation of the complex (coloured blue for non-polar atoms and yellow for polar atoms). Each binding surface is divided into two regions, one predominantly hydrophobic and one predominantly polar.

The hydrophobic region of the interface consists of a ridge of non-polar residues (F44, L45 and F47 (single-letter amino-acid code with residue number)) protruding from the loop between strands 1 and 2 of the SEB molecule (Fig. 4b, blue; and Fig. 4d), which fits into a predominantly hydrophobic depression on the DR1 molecule formed by residues of loops 1 and 3 and the α -helix of the α -chain (Fig. 4a, blue; Figs 3c and 4c). DR1 residues that contribute to this interaction include hydrophobic residues Y13, M36, A37, L60, I63 and A64, as well as the aliphatic portions of more polar residues such as Q18 (Fig. 4c). Mutations of residues F44 and L45 in the SEB molecule disrupt binding to class II MHC molecules²⁴.

The polar region of the binding interface is more apparent on the SEB interaction surface, to the left of the hydrophobic ridge (Fig. 4b). This polar pocket on the SEB surface is complemented by a protrusion from loop 3 of the DR1 molecule formed by lysine 39 (Fig. 4a, e). This lysine forms a completely buried salt bridge with glutamic acid at position 67 of SEB, surrounded by hydrogen bonds from SEB Y89 and Y115 (Fig. 4e). The mutation of lysine 39 to alanine in the DR1 molecule reduces SEB binding⁴⁰.

Three other residues of HLA-DR1 (Y13, Q18 and K67) are potentially involved in hydrogen bonds to the main chain of SEB residues 43–46 (Fig. 4f) and may be important in positioning the hydrophobic residues of the SEB molecule between the DR1 loops. As these residues vary between class II isotypes (Fig. 5), they may play a part in determining differences in the overall binding affinity.

In addition, SEB disulphide loop residues 92–96 contact the α 1-domain α -helix of the DR1 molecule along the upper face of the interaction region (Fig. 4d). In particular, SEB Y94 forms an extensive set of hydrophobic interactions with DR1 residues 60 and 61, whereas SEB S96 contacts DR1 residues 64, 67 and 68. Although these SEB residues do not bind into distinct pockets on the DR1 molecule, they have important implications for the accessibility of DR1 residues to TCR interactions (see below).

Two SEB residues (14 and 17) have been implicated in MHC binding²⁴, but are not directly involved in the DR1–SEB interface and may have an indirect effect on MHC binding. This conclusion is supported by the observation that deletion of SEB residues 1–30 does not abrogate the ability of SEB to stimulate polyclonal T cells²¹.

Other class II molecules and SEB

The binding affinity of SEB varies between different class II

MHC molecules. The ability of SEB to bind many different DR allotypes²⁵ can be explained by its exclusive interaction with the DR1 α -chain, which is conserved in all DR molecules. Binding to other class II isotypes is weaker than binding to DR^{26–29} in the order DR > DQ > DP^{25,29}, whereas for mouse alleles I–E binds SEB better than I–A²⁹, but both bind more weakly than DR.

Figure 5a shows a plot of the surface of the DR1 molecule that is buried by the interaction with SEB, along with the corresponding residues that are found in other human (DP/DQ) and mouse (I–A/I–E) alleles. For the human class II isotypes DP and DQ, about 50% of the residues in the DR1–SEB interface are conserved, although the subset of these residues differs between DP and DQ. Lysine 39 is found in all three isotypes, indicating that a salt bridge with E67 of SEB could be formed (Fig. 4e). Many of the residues that form the hydrophobic portion of the interaction surface (Fig. 4c, and shown in blue in Fig. 4a) are conserved (L60, A64) or conservatively substituted (M36 to L, I63 to I or M).

Although a number of residue differences could account for a lower binding affinity for DQ and DP, relative to DR, the substitution of Q18 to proline in both DQ and DP is particularly central to the DR1–SEB interface. Proline would disrupt one hydrogen bond (Fig. 4e), and would potentially alter the conformation of the other residues in this loop. DP molecules have additional mutations in residues in this region (Y13 to valine and K67 to asparagine) that form one side of the SEB binding site (Figs 3c and 4a, b), which could further destabilize the interaction with SEB molecules.

In the case of the mouse class II molecules, 12 of 17 residues are conserved in I–E molecules and 10 of 17 in I–A molecules. I–E molecules have a lysine-to-serine mutation at position 39, which would abolish the salt bridge with SEB (Figs 4e and 5a). Serine may partially compensate for the lost salt-bridge interaction. Further mutational studies are needed to define the function of different amino acids at the interface.

Other superantigens and class II molecules

Although the sequence similarity of different *S. aureus* toxins with SEB ranges from 40 to 90%, there is evidence for distinct MHC binding sites for different toxins^{28,30,31}. Binding of *S. aureus* enterotoxin A (SEA) has been mapped to the MHC class II β 1 domain^{32,33}; binding of toxic-shock-syndrome toxin TSST-1 has been shown to be sensitive to both α 1 and β 1 domains^{34–36}. A comparison of the SEB residues involved in binding to DR1 with the corresponding residues in SEA and TSST-1 provides some insight into the functional data available for these toxins.

Figure 5b shows a plot of the buried surface area of the SEB residues involved in binding DR1, with the corresponding residues from other *S. aureus* toxins. Of the DR1–SEB interactions described above, a number of central residues are conserved or conservatively substituted in SEA, including F44, L45, D67, Y89 and Y115. The conservation of these residues suggests that SEA may bind to class II molecules in a similar way to SEB. Substitution of the amino acids corresponding to SEB residues F44 and L45 in SEA (F47, L48) reduces its T-cell-stimulatory activity³⁷ and substitution of SEA L48 reduces MHC binding but does not abolish it (J. Kappler, personal communication). Other interactions discussed below may contribute to additional SEA binding.

Figure 5b shows that the major features of the SEB-binding interface are absent in TSST-1, based on a structural alignment of the two proteins^{38,39}. These changes include the loss of the hydrophobic ridge (F44 to S) and the residues that interact with DR1 K39 (SEB E67 to I, Y89 to T, Y115 to I). Mutation of α -chain residues M36 to I, or K39 to S, abolishes TSST-1 binding to HLA-DR7 (ref. 35). Both of these residues are directly involved in the DR1–SEB binding interface (Fig. 4) and mutation of K39 to A disrupts SEB binding as well as TSST-1

binding⁴⁰. This indicates that although SEB and TSST-1 are sensitive to mutations in the same region of the class II α -chain, their specific interactions may be substantially different.

Superantigen residues interacting with TCR

Some mutations in the SEB molecule affect T-cell stimulation, but not class II binding, suggesting specific contacts with the TCR²⁴. These residues are shown in Figure 3a, together with residues that determine V β specificity between enterotoxins SEA and SEE^{2,41,42}. The SEA residues are in the C-terminal domain of the superantigen structure, in a loop between strand 9 and helix 5 of SEB. Mutation of the cysteine residues involved in the disulphide bond in SEA⁴³ and SEB²⁴ also prevents T-cell stimulation. All of these residues line a region between the two domains of the superantigen, defining a potential TCR-binding site that is located above and to one side of the MHC peptide-binding groove (Fig. 3a).

Implications for ternary complex with TCR

The hypervariable region 4 (HV4) of the V β domain of the TCR is important for superantigen interactions^{14–18}. In a hypothetical model of the TCR⁴⁴, this region lies on an exposed face of the V β domain (shown in white in Fig. 3b). Direct binding studies with a soluble TCR β -chain⁴⁵ indicate that these interactions may be sufficient for formation of an MHC–superantigen–TCR complex.

The juxtaposition of the HV4 region of the TCR with the superantigen residues involved in TCR interactions leads to a model with interesting implications for the formation of the ternary complex between MHC, SEB and TCR (Fig. 3b). The complementarity-determining regions (CDRs) of the TCR are oriented over the MHC peptide-binding site, with the V β domain bound to SEB and the V α domain above the class II β 1 domain. This model is consistent with a role for both the TCR α -chain and MHC polymorphism in modulating super-

TABLE 1 Data collection and refinement statistics

TABLE 2. Data collection and refinement statistics						
Data	Cell dimensions (Å)		Resolution (Å)	R_{sym} (%) [*]	Completeness (%)	
Crystal form I, space group $P2_12_12_1$						
Native	95.0 × 114.7 × 149.8		30.0–2.7	5.7	86	
Synchrotron			2.8–2.7	32.1	87	
Crystal form II, space group $P2_12_12_1$						
Native	95.8 × 127.0 × 183.8		30.0–4.35	11.4	82	
GX-13			4.69–4.35	31.0	63	
Refinement statistics						
Resolution (Å)	No. of reflections (working set)	No. of atoms [†]	R.m.s. bonds (Å) [‡]	R.m.s. angles [‡]	R_{cryst} (%) [§]	R_{free} (%)
6.0–2.7	31,557	9,400	0.017	2.16	25.7	32.7

A papain-solubilized form of the human class II MHC molecule, HLA-DR1 (DRA, DRB1#0101)⁵⁸ was co-crystallized with SEB in a 1:1 molar ratio (final total protein concentration, 15 mg ml^{−1}) from a stock solution in 10 mM Tris buffer, pH 7.5. Crystals were grown by vapour diffusion, by mixing 2 μ l protein solution with 2 μ l well solution containing 10 mM sodium acetate, pH 4.7, 10% ethylene glycol, and 12–20% PEG4000 (Fluka), at 25 °C. SEB was obtained as a lyophilized powder from Sigma, or Toxin Technology, from culture supernatants of an SEB-producing strain of *S. aureus* (Toxin Technology) and as a kind gift from M. Sax. Two crystal forms grew under the same conditions. Data for crystal form I were collected from crystals flash-frozen at −165 °C. Data for crystal form II were collected from 8 crystals at 25 °C and merged using the Buddha⁵⁹ and CCP4 programs⁶⁰. Data were collected using a Nicolet/Xentronics area detector and Elliot GX-13 rotating-anode X-ray source with Franks double-mirror optics, and also at the Cornell High Energy Synchrotron Source (CHESS) F-1 beamline, using Kodak phosphor-image plates. CHESS data were processed with Denzo and Scalepack (Z. Otwinowski, personal communication). DR1–SEB SIR/anomalous phased electron density maps were iteratively averaged with DR1–SIR electron density maps and a model of the HLA-DR1 molecule was built as described¹¹. The SEB region was not easily interpretable, so the DR1 model was improved, first by building into single DR1 domain omit maps, comparing maps calculated with both model and experimental SIR/anomalous phases, and maps calculated with DR1 model phases. Cycles of building and refinement of the DR1 model improved the SEB regions and a partial polyaniline model of SEB was built. This partial model of the DR1–SEB structure was used to solve the second, low-resolution DR1:SEB crystal form by molecular replacement using the Navazza suite of programs⁶¹, giving an initial *R*-factor of 40.5% and a correlation coefficient of 48.4% from 8–4.3 Å. Iterative four-fold real-space averaging between the two space groups was carried out using the Bricogne package of programs⁶², generating the electron density map shown in Fig. 1a. A 190-residue polyaniline trace of SEB was built and used to phase five higher-resolution (3.5 Å) maps, where 20% of the SEB polyaniline trace was omitted. Side chains were built into interpretable electron density, and the model was improved by further cycles of building, refinement and iterative two-fold non-crystallographic real-space averaging. The resolution was gradually extended from 3.5 to 2.7 Å. At all stages, refinement paths were taken that minimized the free *R*-factor⁶³. As refinement proceeded, a number of loops in the SEB structure remained untraceable. These loops include residues 57–60 and 99–110 of the first domain and residues 122–126, 176–182, and the N- and C-terminal residues 1 and 236–239 of the second domain. In addition, three regions in the C-terminal domain of SEB do not show unambiguous side-chain density, including the N-terminal SEB region (residues 1–11) β -strands 6 and 7 (residues 127–154), and the C-terminal region of helix 4 and the adjacent loop residues (residues 169–175). In general, density for the second domain is less well defined. Refinement shows a dramatic difference in average atomic *B*-factor values between the HLA-DR1 molecule and the SEB molecule. The average *Ca* *B*-factor is 30 Å² for DR1, 55 Å² for the N-terminal domain of SEB1 (SEB1 is one of the SEB molecules in the asymmetric unit), and 70–80 Å² for the C-terminal domain, showing a radial increase from the DR1–SEB interface (Fig. 1). Given the high temperature factors for SEB, it was important to verify the SEB model. An independently refined model of HLA-DR1 (ref. 20), with no phase bias for the SEB model, was used to calculate $|2F_{\text{obs}} - F_{\text{calc}}|$ averaged and unaveraged omit maps. The omit maps verified the overall placement of the SEB molecule in the map, indicating a deteriorating map quality for SEB regions in the following order: N-terminal domain (SEB1) > C-terminal domain (SEB1) ~ N-terminal domain (SEB2) > C-terminal domain (SEB2), where SEB1 and SEB2 refer to the two SEB molecules in the asymmetric unit. In the final stages, the independently determined structure of the SEC3 molecule was compared to the SEB model, leading to rebuilding of difficult regions of the SEB, including three loops (29–32, 201–203 and 224–229), the N-terminal strand (residues 2–13), and the C-terminal residues of helix 4 and the adjacent loop (157–175). Fewer than 5% of the Φ/Ψ angles lie outside allowed regions of a Ramachandran plot.

^{*} $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where *I* is the observed intensity, and $\langle I \rangle$ is the average intensity from several measurements.

[†] 9,400 non-hydrogen atoms represents 93% of the expected atoms for the DR1–SEB complex. Water molecules have not yet been included in this refinement.

[‡] R.m.s. bond angles and lengths are r.m.s. deviations from ideal values.

[§] *R*-factor calculated with working set reflections greater than 2 σ .

^{||} *R*-factor calculated with 3,383 reflections removed before automated refinement and greater than 2 σ .

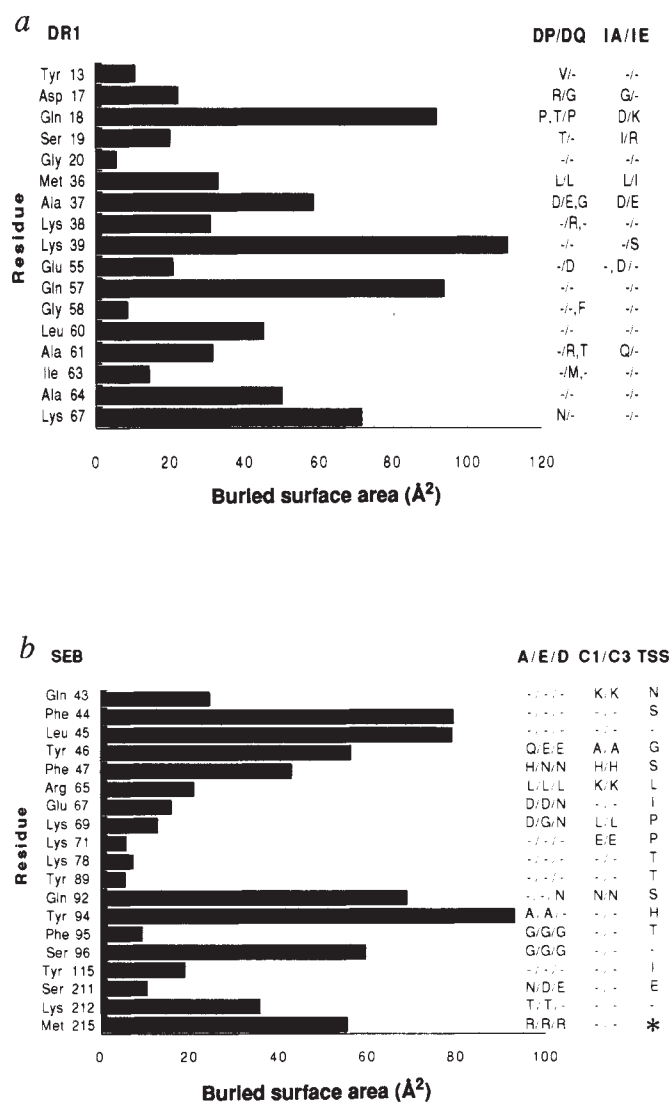


FIG. 5 Residues involved in the DR1-SEB interaction vary between class II isotypes and related superantigens. **a**, Plot of the buried solvent-accessible surface area²² for each residue of the HLA-DR1 molecule involved in SEB binding. Corresponding residues in HLA-DP, HLA-DQ, mouse I-A, and I-E molecules is indicated to the right of the plot. **b**, Plot of the buried solvent-accessible surface area for each residue of the SEB molecule involved in HLA-DR1 binding. Corresponding residues in related superantigens are listed to the right for SEA, SED, SEE, SEC1, SEC3 and TSST-1. The TSST-1 residue alignment is based on the structural alignment of TSST-1 and SEB^{38,39}. Surface areas were calculated with the program Access²² as described in Fig. 4 legend. A dash is used for residues identical to DR1 or SEB and an asterisk indicates a deletion.

antigen stimulation through direct TCR-MHC interactions^{16,24,26,29,46-53}.

However, the DR1-SEB complex suggests that the interactions between TCR and MHC molecules during superantigen stimulation differ from the interactions involved in antigenic peptide stimulation. Residues of the DR1 α 1-domain α -helix that are usually exposed and might interact with the TCR are partially or completely buried in the complex by SEB residues 92-96 (Fig. 3c). These MHC surface residues (at positions 55, 57, 58, 60, 61, 63, 64, 67) influence T-cell stimulation when mutated^{12,13}, indicating that this region of the DR α 1-domain α -helix is important for TCR recognition of peptide antigens and arguing for an unconventional mode of interaction between TCR and MHC during superantigen stimulation.

Discussion

The crystal structure of the DR1-SEB complex shows that SEB binds to the α 1 domain of class II molecules, positioning a TCR-binding site above and to the side of the MHC peptide-binding site. Antigenic peptides are not inhibitors of SEB stimulation¹³ and the structure demonstrates that peptides and SEB occupy two distinct regions of the class II MHC molecule. In the DR1-SEB crystal, electron density is observed for 13 residues of an extended peptide chain, corresponding to a mixture of self peptides bound to DR1 (T.S.J., manuscript in preparation). The

details of the DR1-SEB interaction indicate how superantigen affinity could be modulated for different class II isotypes.

The interaction of related bacterial superantigens with class II molecules may differ from that seen in the DR1-SEB structure. Two lines of evidence support such a view. The first is that different superantigens do not all cross-compete in binding studies. SEB and TSST-1 do not competitively inhibit each other or completely block SEA binding to HLA-DR^{28,30,31}, indicating the existence of independent binding sites. However, SEA is able to compete effectively with both SEB and TSST-1 for binding³¹, suggesting that these sites may overlap. In addition, mutational studies of HLA-DR and SEA suggest that SEA has a different binding site. Histidine 81 of the class II β -chain^{32,33} is important for SEA binding to DR, but has no effect on SEB or TSST-1 binding. This interaction may be mediated by a zinc atom bound to a metal coordination site found in SEA, SED and SEE⁵⁴. SEB does not require zinc to bind class II molecules, and the residues in SEA that bind zinc are in the C-terminal domain, far from the DR1-SEB interface, indicating that SEA has a different mode of binding from SEB.

Important features of the DR1-SEB interaction are conserved in SEA, suggesting that SEA may have evolved two distinct modes of binding to class II molecules, one similar to SEB and another zinc-mediated interaction with the class II β -chain. This is consistent with the available mutational and binding data.

Binding of one SEA molecule to both α -chain and β -chain sites on one class II molecule is unlikely, considering the DRI-SEB structure, the competition data, and the zinc-binding site of SEA. However, one SEA molecule could potentially crosslink two class II molecules, with the N-terminal domain interacting with the $\alpha 1$ domain of class II (as observed for SEB) and the C-terminal domain interacting with the β -chain of another class II molecule. This type of crosslinking might explain the ability of these superantigens to stimulate T cells at concentrations well below their measured K_d values.

The crystal structure also supports a model of ternary complex formation in which the MHC interaction with the TCR is distinct from the peptide-mediated interaction. A stretch of residues located in the SEB disulphide loop lies across the $\alpha 1$ α -helix of DRI, covering residues that have been implicated in TCR recognition of peptide-MHC complexes. This suggests that superantigens may not take advantage of any residual affinity of TCR for MHC derived from positive selection during thymic education. However, the location of SEB residues that interact with TCR and of TCR residues that interact with superantigens

suggests that the TCR may still be positioned in close proximity to the class II peptide-binding site. This may explain how TCR α -chains and MHC polymorphisms can modulate superantigen stimulation. Superantigens may have evolved to bind class II MHC molecules, not in order to use existing MHC-TCR interactions to stimulate T cells, but rather to take advantage of additional signals and organization inherent in conventional antigen presentation.

Superantigens have been directly implicated in a number of diseases and it has been suggested that they might be involved in the generation of autoimmunity⁵⁵ by stimulating existing autoreactive T cells. It has been shown that SEB can induce relapsing paralysis in mice that have previously been immunized with a peptide that induces experimental autoimmune encephalomyelitis^{56,57}. This indicates that the powerful T-cell-activation properties of superantigens may be important in the development and relapse of autoimmune disorders. An understanding of the structural requirements for the action of these superantigens may further the development of strategies to control the onset and progression of disease. □

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Molecular cloud condensation as a tracer of low-mass star formation

Akira Mizuno*, Toshikazu Onishi*, Masahiko Hayashi†, Nagoyashi Ohashi‡, Kazuyoshi Sunada‡, Tetsuo Hasegawa§ & Yasuo Fukui*

* Department of Astrophysics, Nagoya University, Nagoya 464-01, Japan

† Department of Astronomy, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

‡ Nobeyama Radio Observatory, National Astronomical Observatory, Minamimaki-mura, Minamisaku-gun, Nagano 384-13, Japan

§ Institute of Astronomy, University of Tokyo, Mitaka, Tokyo 181, Japan

STARS form inside dense clouds of molecular gas, but the details of the process, such as the quantity of gas that goes into stars and the rate at which the gas collapses, are still unknown. The earliest stages of cloud collapse are particularly poorly understood; some theoretical models exist¹⁻⁴, but there has been no observational evidence to support them. Here we report molecular emission-line data from the Taurus molecular cloud, which allows us to follow the earliest stages of cloud collapse. We find, contrary to previous results⁵, that the cloud cores without young stars are less dense and more extended than those with stars, and that the timescale of core collapse suggested by the data is a few hundred thousand years. This is in good agreement with a model in which the formation rate of low-mass stars is controlled by ambipolar diffusion—the relative drift of neutral molecules with respect to magnetic field lines in the cloud^{1,3}.

Star formation is generally studied by observing emission from trace molecules in the clouds^{6,7}—molecular hydrogen is not observable under most conditions—or using far-infrared emission from the dust^{8,9}. The quasi-static collapse of molecular clouds can be followed by observing molecules that trace different density regions in the clouds. We initially mapped a 5.5 arcdeg² region of the Taurus molecular cloud, observing the emission from C¹⁸O with the Nagoya 4-m telescope¹⁰, which is best for tracing molecular hydrogen densities of $n(\text{H}_2) \approx 10^{3-4} \text{ cm}^{-3}$ (a factor of 10 to 100 greater than the average density of the cloud). In this study, we made high-resolution observations of the C₃H₂ (2₂₀–2₁₁) and H¹³CO⁺ ($J=1-0$) emis-

sion, using the Nobeyama Radio Observatory 45-m telescope from January 1992 to April 1993. These molecules are collisionally excited at densities $n(\text{H}_2) \geq 10^5 \text{ cm}^{-3}$ and are suited to probe the dense cores that are collapsing into stars. We used the fully sampled C¹⁸O map to identify the denser portions of the cloud, which were then observed in the C₃H₂ and H¹³CO⁺ lines.

The regions selected from C¹⁸O data were classified into two categories. One consists of cores with the detected IRAS emission (IRAS cores), which are associated with young stellar objects detected by the Infrared Astronomical Satellite⁹ (IRAS) having 'cold' far-infrared spectra that rise steeply towards longer wavelengths (we refer to IRAS sources with flux density (F) ratios of $\log(F(12 \mu\text{m})/F(25 \mu\text{m})) < -0.4$ as 'cold' IRAS sources. This ratio corresponds to a temperature of $\lesssim 150 \text{ K}$, assuming that the dust emissivity varies as $\lambda^{-1.5}$ at these wavelengths¹⁶). The other category consists of cores without IRAS emission; C¹⁸O intensity peaks without IRAS point sources. We randomly chose 21 of the 25 IRAS cores, and 25 of the 45 cores without IRAS emission, respectively for the two categories. Nine of the 21 observed IRAS cores show compact distributions of line intensities in both H¹³CO⁺ and C₃H₂ emission, within 1 arcmin (0.04 pc) of the IRAS point sources, definitely indicating their physical association with these point sources. For the remaining 12 IRAS cores, H¹³CO⁺ and C₃H₂ emissions are weak and/or do not show clear concentration toward the IRAS sources. The far-infrared spectra of the 9 IRAS sources with compact H¹³CO⁺ and C₃H₂ cores are colder than those of the remaining 12 IRAS sources. The far-infrared colours of the nine IRAS cores tend to be characterized by $\log(F(12 \mu\text{m})/F(25 \mu\text{m})) < -0.7$ and $\log(F(25 \mu\text{m})/F(60 \mu\text{m})) < -0.4$, where F denotes the flux density, incoming energy per unit time, area and bandwidth ($\text{erg s}^{-1} \text{ m}^{-2} \text{ Hz}^{-1}$). By contrast, 11 of the remaining 12 IRAS sources show far-infrared colours warmer than the above values. This tendency suggests that the 9 IRAS cores with the compact cores are younger than the other 12 IRAS sources. (Younger protostars have larger amounts of cold circumstellar dust than evolved ones. Thermal emission from such cold dust is dominant in the far-infrared wavelength. Thus, younger protostars tend to show steeper infrared spectra.) For the cores without IRAS emission, 9 of the 25 observed candidates exhibit significant H¹³CO⁺ and C₃H₂ emission, and we completed the mapping to cover their angular extents for 6 of the 9 detected objects. For the remaining three detected cores without IRAS emission, the dense gas distributions are extended beyond 2×2 arcmin (the limits of the maps). For cores with and without IRAS emission, the H¹³CO⁺ intensity distributions, linewidths and sizes are essentially consistent with those of C₃H₂. Similar intensity distri-

FIG. 1 Contour maps of H¹³CO⁺ ($J=1-0$) total intensity of typical dense cores obtained with the Nobeyama 45-m telescope. The number on the map corresponds to the core number listed in Table 1. Upper panels are maps of dense cores without IRAS emission, except for core (7), which is the lower left core in panel (2), (7). Lower panels are dense cores with IRAS emission. The beam size is 20 arcsec at 3 mm wavelength, corresponding to 0.014 pc at the distance of Taurus. Typical r.m.s. noise of the spectra is $\sim 0.15 \text{ K}$ at the velocity resolution of 0.13 km s^{-1} . Contours are from 0.14 K km s^{-1} with a 0.07 K km s^{-1} step. The positions of IRAS sources are indicated as crosses. The reference positions (given as RA(1950), dec.(1950)) of offsets are as follows: core (1), 4 h 15 min 5.4 s, $27^\circ 27' 43''$; core (2) and core (7), 4 h 16 min 35.5 s, $27^\circ 27' 43''$; core (3), 4 h 25 min 34.5 s, $26^\circ 45' 4''$; core (8), 4 h 16 min 53.8 s, $27^\circ 2' 52''$; core (11), 4 h 28 min 40.2 s, $18^\circ 1' 42''$; core (15), 4 h 36 min 49.3 s, $25^\circ 57' 16''$. The half-power beam width (HPBW) is indicated as a circle in panel (15).

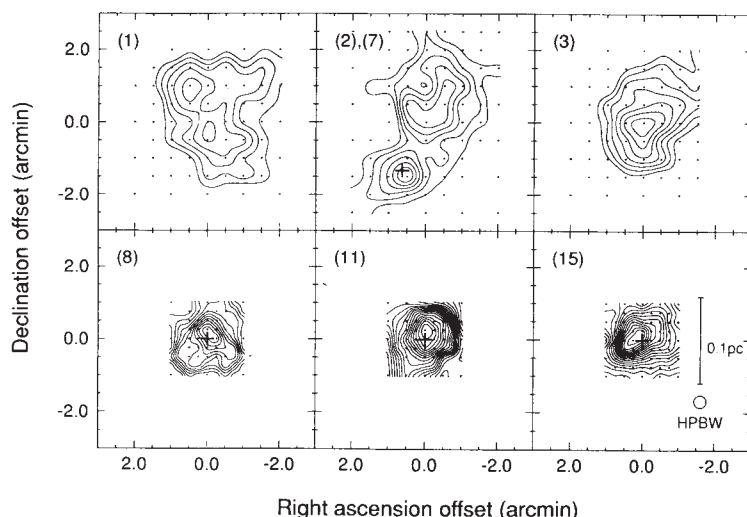


TABLE 1 Physical parameters of H^{13}CO^+ cores

Core	RA (1950)* (h min s)			Dec. (1950)* (° ' ")			$T_A^{\dagger}$ (K)	$\Delta V^{\ddagger}$ (km s ⁻¹)	$R^{\S}$ (pc)	Mass § ($M_{\odot}$)	$n(\text{H}_2)^{\S}$ ($\times 10^5 \text{ cm}^{-3}$)	IRAS source
1	4	15	7.6	27	28	43	1.2	0.35	0.065	1.7	0.25	no
2	4	16	33.2	27	8	13	0.9	0.45	0.053	2.2	0.62	no
3	4	25	34.5	26	45	4	1.1	0.54	0.049	3.0	1.0	no
4	4	28	54.8	24	25	50	0.9	0.54	0.124	7.5	0.16	no
5	4	30	24.0	22	35	46	0.6	0.49	0.129	6.5	0.12	no
6	4	38	35.8	25	53	35	1.0	0.52	0.120	6.8	0.16	no
7	4	16	37.7	27	6	13	1.1	0.49	0.026	1.3	3.0	04166 + 2706
8	4	16	52.7	27	2	52	1.4	0.70	0.029	3.0	5.0	04169 + 2702
9	4	19	10.6	15	23	16	1.1	0.57		—	—	04191 + 1523
10	4	24	52.1	26	12	50	1.1	0.49	0.019	1.0	5.7	04248 + 2612
11	4	28	40.2	18	1	57	1.9	0.78	0.033	4.2	4.8	04287 + 1801
12	4	32	32.4	24	2	15	1.4	¶	0.026	—	—	04325 + 2402
13	4	36	9.8	25	47	30	0.9	0.75		—	—	04361 + 2547
14	4	36	29.0	25	35	56	0.9	0.63	0.033	2.7	3.1	04365 + 2535
15	4	36	49.3	25	57	31	1.8	0.82	0.027	3.8	7.9	04368 + 2557

* Position of H^{13}CO^+ ($J=1-0$) intensity peak. Typical positional uncertainty is $\lesssim 5$ arcsec.

† The results of gaussian fits to the H^{13}CO^+ line profiles at the peak intensity positions. $T_A^{\dagger}$ is a line antenna temperature corrected for atmospheric absorption, and ΔV is a full velocity width at half-maximum.

‡ Geometric mean of the maximum and minimum radii of the dense core. Both radii are determined by a half-maximum intensity contour.

§ Virial mass and average number density calculated from ΔV and R assuming a spherical gas distribution with a radius of R .

|| Half-maximum intensity contour is not sized because of another dense gas component located close to the core.

¶ The line profile is contaminated with another velocity component, and we did not derive the velocity width.

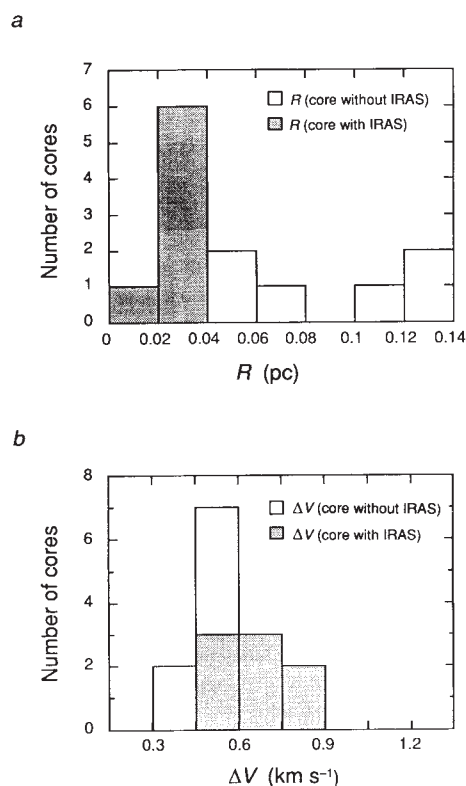
butions in both molecular lines suggest that molecular chemistry does not significantly affect the results of the present study. We shall therefore focus exclusively on the H^{13}CO^+ data in the following discussion.

Figure 1 shows contour maps of the H^{13}CO^+ ($J=1-0$) emission integrated over the velocity range of significant emission between first nulls. In each map, very compact cores smaller than ~ 0.1 pc in radius may be seen. It is remarkable that the IRAS cores (lower row of panels and core 7 in upper panel) tend to be smaller by a factor of ~ 3 , on average, than those of cores without IRAS emission (upper panels except for core 7). This tendency is shown more clearly in Fig. 2a, a histogram of the core radii. We should mention here that a previous study of dense cores in the 1.3-cm line⁵ of NH_3 showed an opposite tendency in core size. This is probably because the beam size at 1.3 cm (~ 1.5 arcmin) was too large to resolve the smaller structure with a size of ~ 30 arcsec. In Table 1, we summarize the physical parameters of the H^{13}CO^+ cores. The average density of the IRAS cores is an order of magnitude greater than that of the cores without IRAS emission, despite the similarity in average masses (2.7 and 4.6 solar masses ($M_{\odot}$), respectively).

FIG. 2 a, Histogram of the radii (R) of dense cores. Shading indicates the cores associated with IRAS sources. b, Histogram of the velocity width (ΔV) of the H^{13}CO^+ molecular line. The velocity widths are determined by fitting gaussian functions to the molecular line profiles obtained at the peak intensity positions. All spectra were obtained with an acousto-optical spectrometer (AOS). The velocity resolution of the AOS is 0.13 km s^{-1} , and the sampling width is about a half of the resolution, that is, Nyquist sampling. A previous NH_3 observation with a 1.5-arcmin beam gave much larger radii, 0.27 parsec on average, for cores with IRAS sources. Such a difference may be because the clustering of cores produces pseudo-cores that show up as a bigger core in the larger beam. The dust temperature decreases obeying a power-law with an index of -0.4 (ref. 15) and T_{dust} is 50–60 K at $r \sim 0.001$ pc (refs 16, 17). This provides an averaged temperature over the beam size (~ 0.013 pc) of ~ 30 K at the centre and ~ 12 K at the periphery $1'$ apart, respectively. According to molecular excitation calculations¹⁸, such a temperature difference causes an increase of the molecular line intensity only by $\sim 30\%$ around density of $\sim 10^5 \text{ cm}^{-3}$. Thus, the intensity distributions are likely dominated not by a local temperature gradient but by density variation. We also note that the virial masses in Table 1 give upper limits, if the cores are dynamically collapsing, making the linewidth larger. These upper limits are perhaps correct within a factor of 2, even if the linewidth increases by $\sim 30\%$ owing to the collapse.

According to numerical studies of the gravitational condensation of dense cores, the internal magnetic fields or turbulent motions of cores dissipate over time, leading to the formation of a protostar^{2,3}. These numerical simulations indicate that the core radius at the point where the integrated intensity is one half of the maximum, decreases in the course of the condensation. The numerical model of Lizano and Shu³ suggests that a core radius becomes smaller by a factor of 3 in $\sim 1 \times 10^6$ yr. The observed trend that IRAS sources are always found in most compact cores is consistent with this picture.

Figure 2b shows a histogram of H^{13}CO^+ line width at full width at half maximum. The average linewidth of the IRAS cores is larger by 30% than that of the cores without IRAS



emission. This may be due to molecular outflow, a mass ejection phenomenon related to the protostellar phase^{11,12,13}, or it may represent gas accelerated gravitationally by the central protostar, either through rotation or accretion. At least four of the nine H^{13}CO^+ IRAS cores have linewidths that sharply increase toward the IRAS position, at a distance of a few tenths of a parsec, suggesting that the increase can be attributed to the gravitational effect of the central protostar. According to the numerical simulation of gravitational collapse for $\sim 1 M_{\odot}$ spherical cores, the infall speed is calculated to be of the order of 1 km s^{-1} at a radius of $\sim 10^{16} \text{ cm}$. The keplerian rotational velocity of a $0.5\text{--}1 M_{\odot}$ star also gives a similar velocity. Higher resolution observations with a millimetre-wave interferometer are necessary to determine which motion dominates the broader linewidth.

An optical spectroscopic study of T Tauri stars indicates that 90% of 73 T Tauri stars have masses below $1.5 M_{\odot}$ (ref. 14). The momentum injected into the surrounding cloud by these low-mass stars is too small to disturb it. In addition, as the total molecular mass is $\sim 6,800 M_{\odot}$, about two orders of magnitude larger than the total stellar mass, star formation activity in one generation should not affect the cloud environment as a whole. Thus, it is likely that star formation in Taurus occurs steadily. We suggest that the H^{13}CO^+ cores without IRAS emission represent cores just before star formation, or in the earliest stage or protostellar collapse, because of their compactness and high densities. C^{18}O cores without H^{13}CO^+ emission are probably the precursors of the H^{13}CO^+ compact cores. Even in the case where protostellar collapse is already initiated in some of the H^{13}CO^+ cores without IRAS emission, the evolutionary stage must be very early and the mass of the protostar small, undetectable at the limit of IRAS sensitivity, 0.1 times the solar luminosity at the distance of the Taurus molecular cloud. As the protostar condenses further, it should become observable at far-infrared wavelengths. Then the compact H^{13}CO^+ cores will appear associated with IRAS cores. The present results indicate that the numbers of cores in the two evolutionary stages are similar (same order of magnitude), but the statistics still need to be improved. We further suggest that the timescales of formation and condensation of H^{13}CO^+ cores are similar to the age of the protostellar IRAS sources (several hundred thousand years). By contrast, there are 70–80 T Tauri stars in Taurus, suggesting that the timescale of the T Tauri phase is five to ten times longer than the evolutionary timescales of H^{13}CO^+ cores. This is consistent with the spectroscopic estimates of the ages of T Tauri stars (a few million years), as determined from the position of the stars on the Hertzsprung–Russell diagram. $\square$

Origin of cometary nuclei as 'rubble piles'

S. J. Weidenschilling

Planetary Science Institute, 620 North Sixth Avenue, Tucson, Arizona 85705-8331, USA

COMETS are extremely fragile objects. Observations of outbursts and splitting have inspired suggestions that their nuclei are 'rubble piles', consisting of components that are weakly bonded, perhaps held together by mutual gravity^{1,2}. This structure was confirmed in spectacular fashion by comet Shoemaker–Levy 9, which disintegrated into about 20 fragments after passing near Jupiter; the tidal stresses induced by the planet were only of the order of 10^{-4} bar (ref. 3). Attempts to explain the formation of comets in the outer Solar System have emphasized either collisional coagulation¹ or gravitational collapse of a layer of dust particles^{4,5}. Here I argue that the observed sizes and structure of comet nuclei are better explained by a two-stage process, involving elements of both models—collisional coagulation in the solar nebula, followed by gravitational instability of a layer of macroscopic bodies.

Planetesimals form after particles settle to the central plane of the gaseous solar nebula. If the particle layer attains a critical density, it can fragment into gravitationally bound condensations⁶. Goldreich and Ward⁷ suggested direct formation of planetesimals from microscopic dust grains. But such a process is impossible due to the nebular gas, which is supported by a radial pressure gradient, and rotates at slightly less than the local Kepler velocity⁸. A dense layer of particles tends to establish keplerian rotation, carrying with it the gas in its vicinity. The resulting shear causes turbulence that prevents the particle layer from settling to the density required for gravitational instability until collisional coagulation produces bodies large enough to decouple from the gas^{9,10}. Conditions are more favourable for gravitational instability in the outer part of the solar nebula than in the terrestrial zone due to three effects: (1) lower critical density due to the smaller tidal effect of the Sun, (2) greater solids/gas ratio due to condensation of ices, and (3), lower gas density which lessens the effects of drag.

A necessary (but not sufficient) condition for gravitational instability is that the particle layer attain a critical density, δ^* , such that its self-gravity exceeds the solar tidal force:

$$\delta^* \approx 3\Omega^2/2\pi G$$

where $\Omega = (GM_{\odot}/a^3)^{1/2}$ is the Kepler frequency (G is the gravitational constant, $M_{\odot}$ is the solar mass and a is the distance from the Sun). The velocity dispersion of the particles must also be small enough to allow density perturbations to grow. The critical velocity, c^* , depends on the surface density of the particle layer, σ_p :

$$c^* = \pi G \sigma_p / \Omega$$

If particles had isotropic random motions, the layer's thickness would be $h \approx c/\Omega$ (ref. 7), making these conditions equivalent. However, particles have systematic radial velocities due to orbital decay induced by drag of the nebular gas. These motions can inhibit gravitational collapse, even when the particle layer has a density much greater than δ^* . The radial velocity is

$$V_r = 2a(\Delta V/V_k)/t_e$$

where ΔV is the deviation of the pressure-supported gas from the Kepler velocity V_k , and t_e is a response time of the body to the drag force⁸. For objects smaller than hundreds of metres in size, V_r is greater than the random motions due to shear-induced turbulence or mutual gravitational perturbations. If all bodies were identical in size (and hence t_e), this systematic motion

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would produce no relative velocities, but more plausibly there would be some range of sizes. A sufficient condition for the dispersion to be less than c^* is for most of the mass to be in bodies large enough so $V_r < c^*$. If the mean free path of a gas molecule exceeds the particle size, the Epstein drag law gives $t_c = d\rho_p/2\rho\bar{v}$, where d , ρ_p are the particle's diameter and density, and ρ , $\bar{v}$ are the density and mean thermal velocity of the gas. For bodies larger than the mean free path, the Stokes drag law gives $t_c = d^2\rho_p/18\eta$, where η is the viscosity of the gas. The condition $V_r < c^*$ requires $d > d^*$, where

$$d^* = \frac{4\Delta V\rho\bar{v}}{\pi G\rho_p\sigma_p}$$

for Epstein drag, or

$$d^* = \left(\frac{\eta\Delta V}{\pi G\rho_p\sigma_p} \right)^{1/2}$$

for Stokes drag. Particles must reach this size by collisional coagulation before gravitational instability can occur.

As an example, I use numerical parameters for Hayashi's¹¹ nebular model, for which $\sigma_p \propto a^{-3/2}$, $\Delta V = 54 \text{ m s}^{-1}$ and $c^* \approx 70 \text{ cm s}^{-1}$ at all values of a . For $\rho_p = 0.7 \text{ g cm}^{-3}$, d^* ranges from $\sim 45 \text{ m}$ at 5 astronomical units (AU) to 80 m at 10 AU (Stokes drag) (1 AU is the mean distance from the Earth to the Sun). Beyond 15 AU, Epstein drag is appropriate, with $d^* \approx 50 \text{ m}$ at 20 AU, 27 m at 30 AU, and 12 m at 50 AU. These values are for a nebula that contains only the minimum mass needed to make the planetary system, but both ρ and σ_p should be proportional to the nebula's surface density. The critical size should therefore be insensitive to the nebular mass, at least for the Epstein drag regime. All plausible nebular models have a radial pressure gradient and corresponding ΔV , so the requirement of collisional growth to macroscopic size is robust.

Drag-induced velocities result in collisions that can produce rapid growth¹⁰, provided that particles are sufficiently 'sticky'. Small grains can adhere to each other and to larger bodies by surface forces¹². The growth mechanism of macroscopic aggregates is less certain, but they will have significant porosity, allowing collisional energy to be dissipated by compaction (see ref. 13 for further discussion of particle sticking). After particles grow to d^* , the layer first becomes unstable to density perturbations of wavelength

$$\lambda_c = 4\pi^2 G\sigma_p/\Omega^2$$

leading to gravitationally bound condensations of mass $M_c \approx \sigma_p \lambda_c^2$ (ref. 7). At 30 AU, with $\sigma_p = 0.4 \text{ g cm}^{-2}$, $M_c \approx 2.4 \times 10^{23} \text{ g}$, which would correspond to icy bodies $\sim 800 \text{ km}$ in diameter ($\rho_p = 1 \text{ g cm}^{-3}$), much too large to identify with cometary nuclei. However, the rotation of the particle disk gives these initial condensations too much angular momentum to collapse directly to solid bodies. Direct collapse is allowed on length scales smaller than

$$\lambda_0 \approx 4.4\pi^{5/4} G^{3/4} \sigma_p / \rho_p^{1/4} \Omega^{3/2}$$

Goldreich and Ward⁷ suggested that this would yield bodies of mass $m_0 \approx \sigma_p \lambda_0^2$. In the Hayashi nebula, $m_0 = 3 \times 10^{17} \text{ g}$, $d \approx 10 \text{ km}$ throughout the region of ice condensation. The size of such bodies is independent of a , as it would be in any nebula with surface density proportional to $a^{-3/2}$ (ref. 14).

Bodies $\sim 10 \text{ km}$ are within the size range of known comets, but may be a few times larger than a typical comet¹⁵. The expression for m_0 implies that a column of material of cross-section λ_0^2 , and vertical dimension equal to the thickness of the particle layer, h , collapses to a single body. However, λ_0 may be significantly less than h . Suppose that the particle layer attains a density $\chi (\geq 1)$ times δ^* before the velocity dispersion becomes small enough for gravitational instability. Then $h = \sigma_p/\chi\delta^*$, and one can show that $h/\lambda_0 \approx 4\rho_p^{1/4} a^{3/4}/\chi$, if ρ_p is in g cm^{-3} and a is in AU; $h/\lambda_0 \approx 50/\chi$ at 30 AU. A narrow, self-gravitating

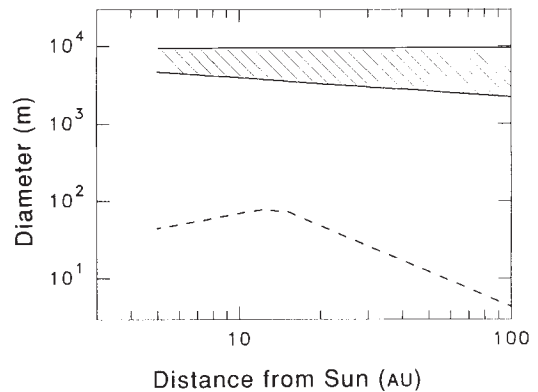


FIG. 1 Sizes of collisionally accreted components (dashed line) and primary nuclei formed by gravitational instability (hatched region) as functions of the distance from the Sun in Hayashi's nebula model (see text). The lower bound of the hatched region assumes that gravitational instability occurs at the minimum critical density δ^* . The upper bound corresponds to mass $\sigma_p \lambda_0^2$, where σ_p is the surface density of particles and λ_0 the maximum length scale for gravitational collapse.

column of material would tend to break up into a series of clumps of roughly equal dimensions, each of mass $(\lambda_0/h)m_0 \approx \lambda_0^3 \sigma_p/h \approx \lambda_0^3 \chi \delta^*$. For the nominal parameters at 30 AU, this yields a typical mass $\sim 7 \times 10^{15} \text{ g}$, or $d \approx 3\chi^{1/3} \text{ km}$. The actual value of χ will depend on such variables as the sizes of the particles and the degree of turbulence generated by shear and any other sources. Still, unless the particle layer reaches a density $\gg \delta^*$, the bodies formed will be smaller than 10 km . I refer to these bodies formed by gravitational collapse as primary nuclei. Figure 1 shows the expected sizes of the primary nuclei and their collisionally accreted component blocks as functions of a .

We now have a general picture of comet formation: icy grains settle to the central plane of the nebula. The particle layer becomes turbulent due to shear, and experiences a period of collisional accretion until bodies reach a mean size of tens of metres. Diminishing relative velocities due to decoupling from the gas allows gravitational collapse to form nuclei a few kilometres in size, consisting of $\sim 10^6$ collisionally accreted 'building blocks'. These nuclei in turn are initially in gravitationally bound clusters of mass M_c , containing $\sim 10^7$ comet-sized bodies. Goldreich and Ward⁷ showed that at 1 AU gas drag would damp internal motions of such clusters and cause their collapse on a timescale $\sim 10^3 \text{ yr}$. However, the much lower gas densities in the outer nebula produce damping times $> 10^6 \text{ yr}$ at 30 AU. On this timescale, the clusters evolve by collisions among the primary nuclei. Large bodies will accrete near the centres of the clusters; these may form the population of Pluto- and Triton-like bodies inferred by Stern¹⁶, and perhaps embryonic cores of the giant planets. Their perturbations, and encounters with other clusters, will cause some fraction of the comet-sized primary bodies to escape from the clusters.

There is some observational evidence of a lower cutoff in the size distribution of comets, with absolute numbers decreasing at diameters less than a few kilometres^{15,17}. A purely collisional origin (by either accretion or fragmentation), would tend to produce a power-law distribution at all sizes. Origin by gravitational instability should lead to a deficit of bodies smaller than kilometre scale. Larger bodies (for example, Chiron) could form by accretion of the primary nuclei. Some smaller bodies could be produced by collisional fragmentation, or tidal disruption by encounters with the outer planets, but the size signature of the primary bodies could survive.

This model for the origin of comets has significant implications for their structure and composition. During the collisional growth phase, relative velocities are comparable to ΔV (tens of

metres per second). The 'building blocks' that accrete in this fashion should be fairly compact in structure, with porosity on small size scales. Primary nuclei assembled by gravitational instability at lower relative velocities ($<c^*$) should retain large spaces among these blocks, resulting in a low overall density. A typical nucleus may contain comparable fractions of volume in both microscopic pores and a network of large interconnected voids. The bimodal distribution of porosity may affect the thermal structure of the nucleus and its permeability to escaping volatiles. While this model has some similarity to the fractal structure proposed by Donn and Hughes¹⁸, it differs by having a preferred size scale for the component blocks.

During the coagulation stage, drag-induced orbital decay can produce substantial radial migration. A typical block may move inward by $\sim 10\%$ of its original distance from the Sun¹⁹. There should be a comparable dispersion of the migration distance among different bodies, thus a given nucleus may contain material derived from initial distances over a range of a few AU. A cometary outburst typically arises from a localized region of the nucleus, and involves a mass of material corresponding to a spherical volume of ~ 20 – 100 m diameter^{20–22}. This is comparable to the predicted size of a component block, strongly suggesting that each outburst is associated with a single block, perhaps of anomalously volatile composition (for example, CO) derived from a larger heliocentric distance. Other possible mechanisms of outbursts are polymerization of HCN (ref. 22), or conversion of amorphous H₂O to crystalline ice²³. Both of these processes are exothermic. The macroscopic voids and small contact area would allow a single block to convert in phase without 'igniting' the transition to its neighbours. The episodic nature of outbursts may be due to this structure, in which case amorphous H₂O need not be a rare constituent present only as isolated 'pockets' within cometary nuclei. The sizes of primary nuclei do not vary strongly with heliocentric distance, but their component blocks are smaller at larger distances; the relative sizes of outbursts among different comets may be correlated with the sites of their formation.

The sizes of the fragments of comet Shoemaker–Levy 9 are believed to be $\sim 10^2$ – 10^3 m, too large to be individual blocks accreted in a plausible nebula. M. Mumma (personal communication) suggests that the parent body was itself a composite of ~ 20 primary nuclei, each of which corresponds to one of the visible fragments. Although they are smaller than predicted, this cannot be ruled out, as the details of the gravitational collapse process are uncertain. It is more likely that the comet was a single primary nucleus, with fragment sizes determined by tidal stresses and sticking forces between the components. In either case, the breakup should have released an extended swarm of bodies tens of metres in size, as well as the visible dust. The fragments of Shoemaker–Levy 9 will strike Jupiter in July 1994. Although the large pieces will all strike the far side of the planet, the atmospheric entry of ~ 10 -m bolides may be visible at its limb. The visible fragments may also be subject to further tidal disruption during their final approach. Observers should watch for unusual activity shortly before each fragment vanishes behind the planet. Each impact may resemble a shotgun blast rather than a rifle shot, with possibly observable consequences for the deposition of mass and energy in Jupiter's atmosphere. □

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A relict refractory inclusion in a ferromagnesian chondrule from the Allende meteorite

Keiji Misawa*† & Takashi Fujita‡

* Department of Antarctic Meteorites, National Institute of Polar Research, 1-9-10, Kaga, Itabashi, Tokyo 173, Japan

‡ Department of Geology and Mineralogy, Faculty of Science, Kyoto University, Sakyo, Kyoto 606-01, Japan

CARBONACEOUS chondrites such as the Allende meteorite are composed of abundant ferromagnesian chondrules and (Ca, Al)-rich inclusions (CAIs) embedded in a fine-grained matrix. The chondrules were formed by melting of pre-existing solid precursor materials¹, whereas CAIs formed either by direct condensation from the nebular gas or by high-temperature processing of solids in the nebula². These two components usually appear as discrete objects in chondrites, and are believed³ to have formed independently before agglomeration of the chondrite parent bodies. However, elemental analyses of some Al-rich chondrules^{4–8} suggest that there was a refractory component—such as CAIs—in the chondrule precursor material. We report here the discovery of a ferromagnesian chondrule from the Allende meteorite that contains an intact CAI fragment. This finding confirms that some CAIs co-existed with the precursor material of ferromagnesian chondrules, and helps to constrain the timing of the formation events of these two components.

Barred olivine chondrule R-11, which was found in the Allende (CV: Vigarano subtype) meteorite, consists mainly of zoned olivine (fayalite 7–18 mol%; Mg-rich core and Fe-rich rim), low-Ca pyroxene, augite, plagioclase (anorthite ~ 83 mol%) and alkali-rich glass with minor amounts of metallic Fe and Fe–Ni sulphides, and can be assigned to a ferromagnesian type⁹. The most characteristic feature of the chondrule is that a coarse, subhedral spinel grain (up to 200 μ m in size) containing silicate inclusions and tiny metallic grains is embedded in a central portion of the chondrule (Fig. 1a). The silicate inclusions consist of Ti, Al-pyroxene (fassaite) and a Ca, Al-rich phase (Table 1 and Fig. 1b). The submicrometre-sized grains are composed of refractory platinum metals (RPMs) with minor amounts of Fe and Ni. This is the first detected occurrence of RPM nuggets inside a ferromagnesian chondrule from the Allende meteorite. Sheng *et al.*¹⁰ described FeO-poor spinel grains in chondrules (they refer to the objects as plagioclase-

† Present address: Department of Earth and Planetary Sciences, Faculty of Science, Kobe University, Nada, Kobe 657, Japan.

TABLE 1 Representative analyses of phases in Allende barred olivine chondrule R-11

	Spinel*		Fassaite†		Olivine		Pyroxene*		Plagioclase†	Glass*
	Core§	Rim§	(2 points average)		Core†	Rim*	Low-Ca	Augite		
SiO ₂	NA	NA	41.7	37.7	41.5	37.8	56.5	53.5	45.8	42.4
TiO ₂	0.19	0.20	2.60	0.24	0.41	ND	0.65	1.23	ND	0.08
Al ₂ O ₃	70.2	67.4	18.6	20.9	0.18	2.08	2.23	3.62	32.7	33.0
MgO	28.8	26.5	9.35	1.94	52.5	42.4	35.2	24.0	0.60	1.01
FeO	0.34	3.20	0.17	0.19	4.83	16.4	0.83	0.57	0.56	0.42
MnO	0.08	0.13	NA	NA	0.08	ND	0.20	0.19	0.05	0.07
CaO	NA	NA	25.6	38.5	0.34	0.18	3.17	16.6	18.1	4.32
Cr ₂ O ₃	0.31	0.31	NA	NA	0.35	0.31	0.97	0.97	ND	0.08
Na ₂ O	NA	NA	NA	NA	ND	NA	NA	NA	2.07	15.7
K ₂ O	NA	NA	NA	NA	ND	0.05	ND	0.08	0.03	2.79

All values are given in wt%; NA and ND, not analysed and not detected, respectively.

* Analysed using a Hitachi S-530 scanning electron microscope equipped with an energy dispersive spectrometer (Kevex Delta System). An accelerating voltage is 20 kV for both oxide and silicates. Data were corrected by a ZAF method.

† Analysed using a JEOL JXA-8800 electron probe microanalyser operated at an accelerating voltage of 15 kV and a beam current of 17 nA. Data were corrected by a ZAF method.

‡ Because of the difficulty of contamination-free analyses, we refer to this as a 'Ca, Al-rich phase'.

§ V₂O₅ contents of the spinel core and the rim are 0.38 and 0.35 wt%, respectively.

olivine inclusions) from four carbonaceous chondrites. However, these spinels are much finer than the R-11 spinel and contain neither silicate inclusions nor RPM nuggets.

The spinel is a relict phase because it is not ordinarily found in mafic silicate chondrules in the Allende meteorite. As shown in Fig. 2, the spinel exhibits Fe-Mg zoning such that an interior portion (as well as the silicate inclusions) is iron-free, but in the rim (~20–40 µm width) and along the cracks the FeO concentration rises sharply. This zoning may have been generated by diffusional replacement of Mg in the spinel and Fe in a surrounding liquid during chondrule-formation melting. Because the silicate inclusions and the RPM nuggets were seen completely enclosed by the spinel (Figs 1, 2), they were also considered to be relict phases. Compared with the conditions of formation of the spinel containing refractory silicate inclusions and RPM nuggets, most precursor materials of the host chondrule may have formed under more oxidized and/or low-temperature environments.

The relict phase assemblages (spinel, RPM nugget, fassaite and a Ca, Al-rich phase) are similar to those of CAIs. It is widely accepted that RPM nuggets in CAIs were produced as high-temperature condensates in the early solar nebula^{11–16}. By analogy with formational history of CAIs, we suggest that the spinel associated with RPM nuggets and silicate inclusions in chondrule R-11 is a relict fragment of high-temperature condensates from the nebular gas (that is, CAIs). The spinel could have escaped complete melting either because of its higher liquidus temperature or because the timescale of heating and cooling of the host barred-olivine chondrule was so brief that the coarse spinel grain incompletely dissolved in a chondrule melt. The barred-olivine textures have been reproduced in experimental studies over melting temperatures in the range 1,500–1,700 °C and cooling rates of 500–2,300 °C per hour^{17,18}.

Relative to cosmic proportions of the elements (here we use the most primitive, CI-chondrite Orgueil values¹⁹ for normalization, and re-normalized to Os=1.0 in Fig. 3), the W, Ir and Ru abundances of the R-11 nugget are identical to those of a type A CAI²⁰. A slight depletion of Mo relative to other RPMs of similar volatility (Os, Ir, Ru) may have occurred during condensation of the nugget in the nebula at high oxygen fugacities¹⁵. Abundances of Pt and Rh (the least refractory among RPMs), and Ni and Fe (less refractory siderophiles) decrease with increasing volatility, suggesting that the nugget may have been isolated from the nebular gas before all of the RPMs and Fe-Ni could condense (~1,200 °C at 10⁻³ atm)^{12,14,16}. The RPM nuggets in R-11 are similar both in size and chemistry to those in type A CAIs²⁰, but quite different from larger opaque assemblages (named Fremdlinge¹³) of Os-Ru grains, metallic Fe-Ni,

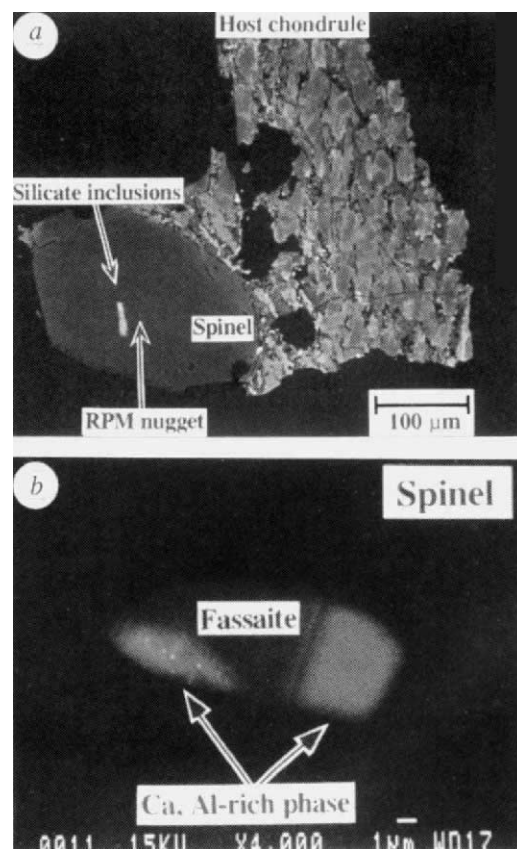
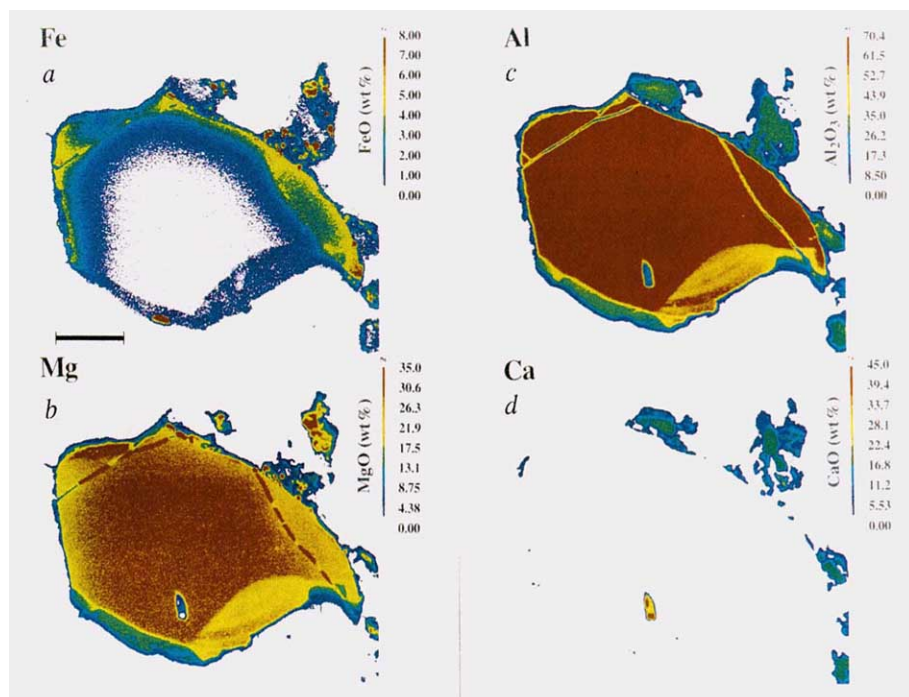


FIG. 1 Back-scattered electron images of Allende barred olivine chondrule R-11. *a*, Chondrule R-11 consists mainly of olivine, low-Ca pyroxene, augite, plagioclase and alkali-rich glass, and can be assigned to a ferromagnesian type³. A coarse, subhedral spinel grain containing refractory platinum-group metal (RPM) nuggets and silicate inclusions (light-grey area) is embedded in the chondrule. The RPM nugget quantitatively analysed in this study (Fig. 3) was lost by polishing. During the course of stepwise polishing of the chondrule, however, we found more than 15 metallic nuggets which were heterogeneously distributed in the spinel. Partly polished section, dark area is epoxy resin. (Scale bar, 100 µm.) *b*, Enlarged view of the silicate inclusions shown in *a*. Light-grey area is a Ca, Al-rich phase and dark-grey area is Ti, Al-rich pyroxene, fassaite (Table 1). Several submicrometre-sized blebs (containing heavy elements) may be seen in the Ca, Al-rich phase. Dark area is spinel. Scale bar, 1 µm.

FIG. 2 Fe (a), Mg (b), Al (c) and Ca (d) X-ray images of the R-11 spinel obtained by a microstep mapping technique using an electron probe microanalyser (JEOL JXA-800). Each point was counted for 40 ms and 500 × 400 points were measured at 0.5-μm intervals. Concentration gradients in a lower portion of the spinel (10–30 μm width of yellow to blue area seen in the Al map, c) are artefacts mainly due to edge effects and contamination. The spinel exhibits Fe–Mg zoning: the core and the Ca, Al-rich silicate inclusions are iron-free, whereas in the rim (~20–40 μm width) and along the cracks the FeO concentration of two phases: a Ca, Al-rich phase (low MgO content) and Ti, Al-pyroxene, fassaite. All four X-ray images are to the same scale (scale bar shown in a, 50 μm).



magnetite (Fe_3O_4) and sulphides found in type B CAIs^{16,21,22}.

The relict spinel and silicates show similarities and differences in comparison with 'fluffy' type A CAIs (FTAs) which are considered to be high-temperature nebular condensates^{23,24}. In the spinel core, TiO_2 , Cr_2O_3 and V_2O_5 contents do not exceed 0.5 wt%, which is different from the V-rich nature of some spinel grains in FTAs^{23,24}, but similar to spinels in type B CAIs² and in Murchison (CM: Mighei subtype) CAIs²⁵. Spinel in Allende FTAs rarely contain silicate inclusions, whereas spinels in Murchison CAIs sometimes contain 2–10-μm inclusions of anhedral Al-diopside, associated with circular voids^{25,26}. Fassaite in R-11 is similar in composition to those in FTAs^{23,24} and is within the range of pyroxene compositions of both type B and

type C CAIs^{2,4}. Because the relict phases in R-11 may have been protected from an open-system nebular alteration^{23,27,28} and from low-temperature oxidization and sulphidization^{21,22}, their composition is more primitive than that of altered CAIs found in the Allende meteorite. We conclude, therefore, that relatively unaltered primitive nebular condensates were preserved inside the ferromagnesian chondrule, although they have modified to some degree by chondrule-formation melting.

There exist Al-rich chondrules in Allende and in other meteorites whose bulk chemistry is more Ca, Al-rich compared with typical magnesian silicate chondrules⁴. The existence of chondrules intermediate in composition between the Al-rich type and the ferromagnesian type suggests that they formed by related processes. Some Al-rich chondrules show highly fractionated abundance patterns of rare-earth elements similar to those previously found only in CAIs, indicating that they contained a small proportion of some of the same refractory precursor components as CAIs^{5–8}. However, there is no direct evidence that chondrule precursors and CAIs have been in the same nebular region. Chondrule R-11 is the first case that a CAI and a ferromagnesian chondrule were mechanically joined together. High-temperature condensates (CAIs) must have formed near the nebular location of chondrule formation. They could have been swept into a high oxygen fugacity and/or a low-temperature nebular region, and have been later incorporated by the precursor materials of the more common ferromagnesian chondrules before chondrule formation. Alternatively, CAIs and precursor materials of ferromagnesian chondrules could have been formed in the same region of the solar nebula but at different stages, which in turn implies that the physico-chemical conditions of the nebula in this region changed dramatically between the two formational events. The implication is that Allende CAIs and ferromagnesian chondrules are more closely related than previously thought, and that they must have formed at different times: the formation of CAIs preceded that of ferromagnesian chondrules. □

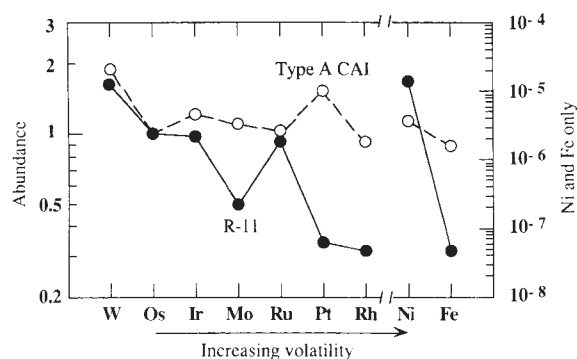


FIG. 3 Abundance of refractory platinum-group metals (RPM, left scale) relative to CI-chondrite (Os=1.0), and of Ni and Fe (right scale) in the R-11 nugget. The abundances are relative to the CI-chondrite Orgueil¹⁹, and re-normalized to Os=1.0. Data for a typical Type A CAI nugget²⁰ are shown for comparison. Elements are arranged in the order of increasing volatility^{12,14,16} (expected from a solar composition) from left to right. The nugget was analysed using a scanning electron microscope (Hitachi S-530) equipped with an energy dispersive spectrometer (Kevex Delta System). The accelerating voltage was 20 kV and data were corrected by a ZAF method. Pure RPMs were used as standards. Analytical results (normalised to total 100% after ignoring Mg, Al and Fe contributions to the analyses from contamination of the spinel) are: Fe, 0.32; Ni, 5.38; Mo, 16.7; Ru, 24.0; Rh, 1.53; W, 5.43; Os, 17.5; Ir, 16.8; Pt, 12.0 (all wt%). Analytical precision is considered to be ~10%.

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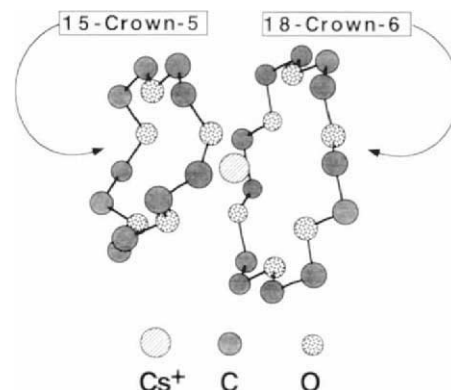
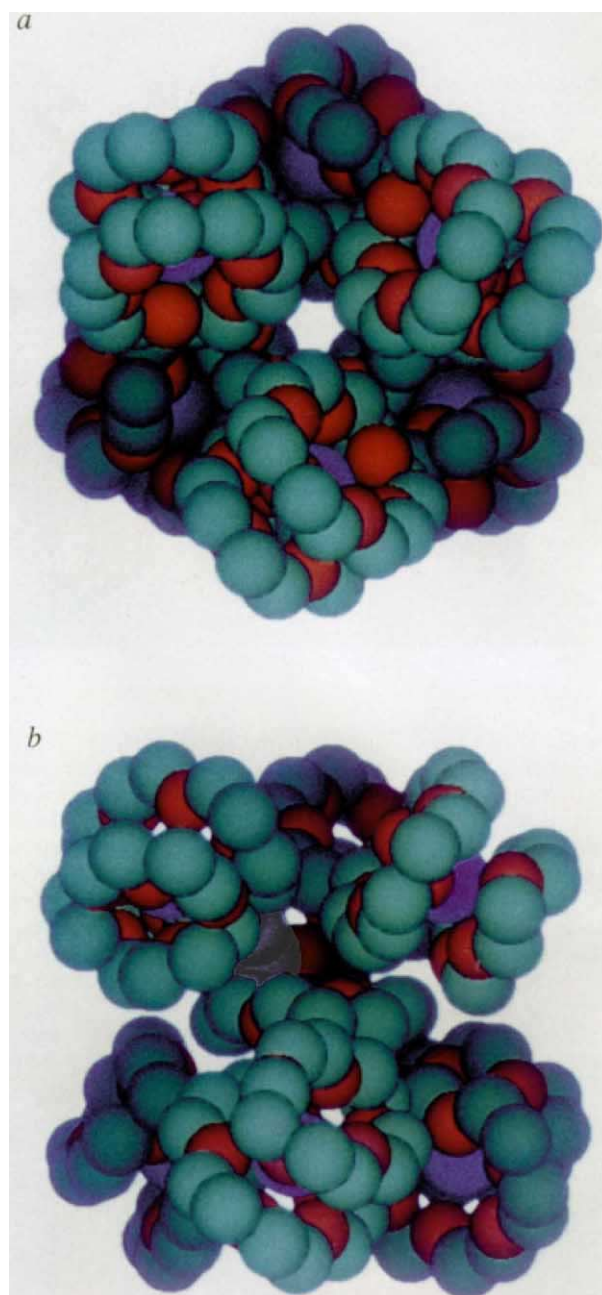
An electrider with a large six-electron ring

Michael J. Wagner*, Rui H. Huang*,
Judith L. Eglin*† & James L. Dye*‡

* Department of Chemistry and Center for Fundamental Materials Research, Michigan State University, East Lansing, Michigan 48824-1322, USA

ELECTRIDES are crystalline salts that contain complexed alkali metal cations whose charge is balanced by trapped electrons¹. Theory^{2,3} and experiment^{4,5} indicate that the excess electron distribution is concentrated in cavities and channels formed by close-packing of the large complexed cations. Thus electrideres might serve as models of a confined electron gas. Only three electrideres have been structurally characterized previously^{6–8}. Here we report the structure of a new electrider, $[\text{Cs}^+(15\text{C5})(18\text{C6})\cdot\text{e}^-]_6\cdot(18\text{C6})$, where 15C5 and 18C6 represent crown ethers with five and six oxygen atoms respectively. The unit cell has threefold symmetry, with a central 18C6 molecule surrounded by six Cs^+ cations, each sandwiched between a 15C5 and 18C6 molecule. The six electrons released from the Cs/crown ether interaction seem to be trapped in six cavities which form a puckered ring, three above and three below the plane of the central 18C6 molecule. The ground state is diamagnetic. This ring-like distribution of electrons contrasts with the chain-like connections between electron cavities observed in other electrideres^{6–8}. Polycrystalline samples of this new electrider have an electrical conductivity about a million times greater than those of the electrideres $\text{Cs}^+(15\text{C5})_2\cdot\text{e}^-$ and $\text{Cs}^+(18\text{C6})_2\cdot\text{e}^-$. The size, shape and connectivity of the electron-containing cavities and channels evidently exert a critical influence on the properties of electrideres.

The new mixed-sandwich electrider has the empirical formula $[\text{Cs}(15\text{C5})(18\text{C6})]_6\cdot 18\text{C6}$ (although it also contains some excess



† Present address: Department of Chemistry, Mississippi State University, Mississippi State, Mississippi 39762, USA.

‡ To whom correspondence should be addressed.

TABLE 1 Crystal data for $[\text{Cs}^+(15\text{C}5)(18\text{C}6)\cdot\text{e}^-]_6(18\text{C}6)$ at 180 K

Space group	$R\bar{3}$ (148)																			
Cell parameters	$a = b = 33.108$ (6) Å $c = 16.266$ (5) Å $z = 18$																			
Range of 2θ	3.5° to 45°																			
No. of reflections used in refinement	2,905 with $F_o^2 > 3.0\sigma(F_o^2)$																			
No. of variables	333																			
Unweighted agreement factor (R)	0.050																			
Weighted agreement factor (R_w)	0.031																			
High peak in final difference map	0.48 e Å^{-3}																			
Selected positional parameters (inversion centre at 0.0, 0.0, 0.5) and thermal parameters B																				
	<table><tr><td>x</td><td>y</td><td>z</td><td>B</td></tr><tr><td>Sandwiched Cs^-</td><td>0.2803</td><td>0.0324</td><td>0.8336</td><td>3.55 (3)</td></tr><tr><td>Defect Cs^+ (multiplicity 0.047)</td><td>0.0</td><td>0.0</td><td>0.3876</td><td>10.6 (6)</td></tr><tr><td>Trap centre</td><td>0.170</td><td>0.215</td><td>0.660</td><td></td></tr></table>	x	y	z	B	Sandwiched Cs^-	0.2803	0.0324	0.8336	3.55 (3)	Defect Cs^+ (multiplicity 0.047)	0.0	0.0	0.3876	10.6 (6)	Trap centre	0.170	0.215	0.660	
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caesium). It was synthesized by anaerobically dissolving caesium metal and ~10% excess of a 1:1 mixture of 15C5 and 18C6 in dimethyl ether (sometimes with methylamine) in a modified H-cell⁶. The temperature was kept below 220 K to prevent thermal decomposition. Crystals were grown either by slow cooling after adding trimethylamine to reduce the solubility, or by slowly evaporating dimethyl ether from the mixture. Rapid crystallization gave mixtures with $\text{Cs}^+(15\text{C}5)_2\cdot\text{e}^-$ and $\text{Cs}^+(18\text{C}6)_2\cdot\text{e}^-$.

The phase purity of polycrystalline samples was checked by differential scanning calorimetry. The 'parent' electrides, $\text{Cs}^+(15\text{C}5)_2\cdot\text{e}^-$ and $\text{Cs}^+(18\text{C}6)_2\cdot\text{e}^-$, show characteristic endothermic peaks at 266 K and 312 K, respectively, and the onset of exothermic decomposition peaks at 299 K and 315 K. The mixed-sandwich endotherm is at 284 K ($\Delta H = 7\text{ kJ mol}^{-1}$) and decomposition begins at 308 K; both temperatures are between those of the parent compounds.

Two crystals from separate syntheses were selected for X-ray structure determination and were mounted anaerobically in the apparatus at ~220 K by previously described techniques⁹. The crystal structure is complex but highly symmetric. The $R\bar{3}$ space group yields a 3-fold rotation axis (the c axis) perpendicular to the plane of, and through the centre of, a 'free' 18C6 molecule, as well as inversion symmetry through its centre. Table 1 gives some structural data. (For a complete set of atomic coordinates, see Supplementary Information.)

Six mixed-sandwich complexes of Cs^+ , centred 5.4 Å from the plane of the free crown ring, are grouped around the central 18C6 molecule in the shape of a double three-bladed propeller. Each of the three 'blades' above the ring makes an angle of 60° with respect to the plane of the central ring. The three 'blades' below the ring are equivalent, but tilted in the opposite direction. Figure 1 shows two views of the central 18C6 ring and the six 15C5- Cs^+ -18C6 sandwiches. These complete units pack together efficiently, but leave empty cavities and channels in which we expect to find the excess electrons.

The irregularly shaped cavities may be approximated as ellipsoids (~5 Å along the c direction and 2.5 Å in diameter). The collection of six cavities, with the overall symmetry of the space group, forms a puckered six-membered ring as shown schematically in Fig. 2. The cavities are alternately above and below the plane of the central 18C6 molecule and each is connected to its

two neighbours by rather open channels with centre-to-centre distances of ~8 Å. In addition, each cavity has two longer channels to neighbouring six-cavity rings. The 'top' cavities connect to 'top' cavities of neighbouring rings through both channels with centre-to-centre distances of ~10 Å, and similarly for the bottom cavities. We presume that one electron occupies each cavity, and that intra-ring electron-electron coupling is the strongest. Inter-ring coupling could, however, provide a three-dimensional conducting network of cavities and channels.

Although the structure was essentially solved on the basis of the description given above, the difference map of electron density showed distinct spherical peaks, ~1.8 Å above and below the central 18C6 ring, that were ~10 times the background level. The diameter of each of these spherical regions was ~2 Å, large enough to accommodate a Cs^+ ion. A probable location for excess Cs^+ would be in contact with the free 18C6 molecule at its centre, with a Cs-O distance of ~3.5 Å and located ~1.6 Å above the centre of the crown ether ring. This is close to the position of the excess electron density (1.8 Å). By including partial occupancy of Cs^+ at these positions, the excess electron density was removed and the agreement factors were improved from $R=0.069$ and $R_w=0.050$ to $R=0.050$ and $R_w=0.031$ (where R and R_w are the unweighted and weighted agreement factors respectively). The refined occupancies for two separately synthesized crystals correspond to 4.5 ± 0.2 and 4.7 ± 0.2 mol per cent excess caesium, so that about one in four of the central 18C6 molecules is coordinated to one Cs^+ ion. Unless a corresponding number of sandwich sites for Cs^+ were empty, the electrons released by the inclusion of excess caesium would be added to the 'pool' of trapped electrons. Thus, caesium acts as an n-dopant.

The electronic contribution to the magnetic susceptibility was measured from 2 to 230 K with an SHE Model 800 SQUID susceptometer. It is strikingly different from those of $\text{Cs}^+(15\text{C}5)_2\cdot\text{e}^-$ and $\text{Cs}^+(18\text{C}6)_2\cdot\text{e}^-$, which both show Curie-Weiss behaviour at high temperatures and weak antiferromagnetism, with maxima in the susceptibilities at 4.5 K and 50 K, respectively^{7,10}. By contrast, the mixed-sandwich electride exhibits weak field-independent paramagnetism that indicates strong inter-electron coupling. The paramagnetism increases with temperature to 3% and 13% of the value expected for free spins at 80 K and 200 K, respectively. The susceptibility below ~50 K increases rapidly with decreasing temperature T , indicating the presence of up to 4-5% of excess electrons. The $T^{-0.6}$ dependence of the 'paramagnetic tail' of a sample with only 0.7% unpaired spins is reminiscent of quasi-one-dimensional behaviour^{11,12}.

The magnetic susceptibility data are qualitatively in accord with a model of diamagnetic six-electron rings weakly coupled together, with 'extra' electrons required to balance the charges of the defect Cs^+ ions on the central 18C6 rings. As expected, the magnitude of this effect varies from sample to sample. Also, the number of unpaired electrons need not be the same as the number of excess Cs^+ ions. Some regions might contain two defects with paired excess electrons, some sandwich cation trapping sites might be empty, and partial decomposition could remove unpaired electrons.

The ^{133}Cs NMR chemical shift of +17 p.p.m. at 210 K is far smaller than the values of +547 p.p.m. and +132 p.p.m. for $\text{Cs}^+(15\text{C}5)_2\cdot\text{e}^-$ and $\text{Cs}^+(18\text{C}6)_2\cdot\text{e}^-$, respectively, and the temperature dependence has the opposite slope. This behaviour is expected from the small value of the paramagnetic susceptibility and its increase with temperature. Even accounting for the low percentage of unpaired spins, however, it is clear that the electron contact density at the caesium nucleus is orders of magnitude lower than in the parent electrides; the mixed-sandwich complex of Cs^+ is much more effective at excluding unpaired electron density.

Both d.c. and a.c. powder conductivities show that the mixed-sandwich electride is at least 10^6 times more conductive than

◀ FIG. 1 Space-filling model of the central $[\text{Cs}^+(15\text{C}5)(18\text{C}6)\cdot\text{e}^-]_6(18\text{C}6)$ unit, constructed from the X-ray coordinates. Colour code: blue, C; red, O; purple, Cs^+ . a, Top view (down the c -axis). Note that the central 'free' 18C6 molecule is hidden in this view. b, Side view (perpendicular to the c axis). c, A 'ball and stick' model of one of the six 15C5- Cs^+ -18C6 sandwich units. In all views the hydrogens have been deleted for clarity.

a

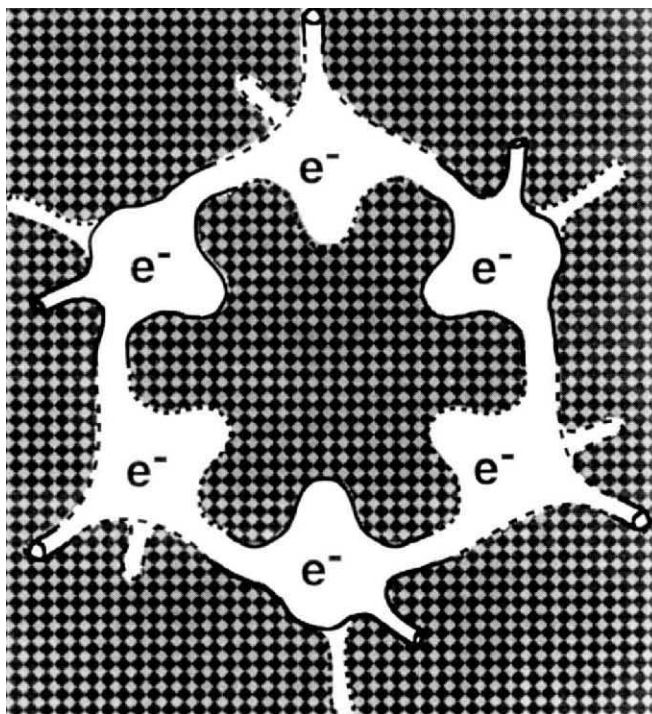
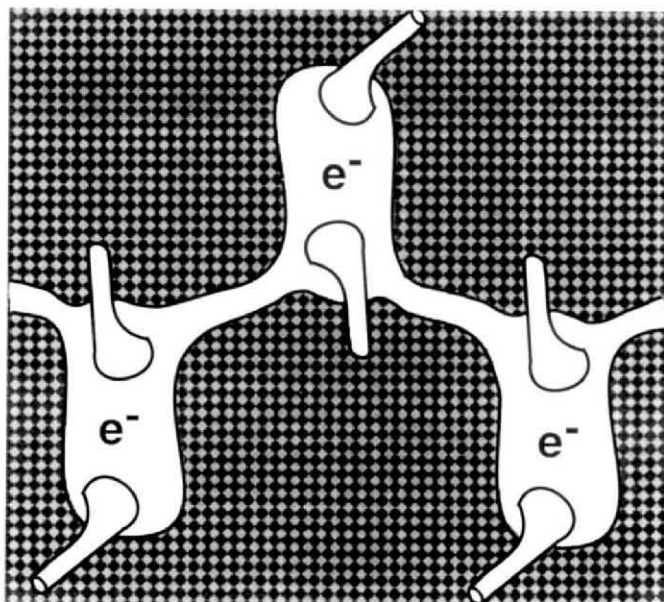


FIG. 2 Schematic diagram of the cavities and channels in the central unit of the electride. *a*, Top view (the solid outline is above the plane of the central 'free' 18C6 molecule while the dashed outline is below).

b



The 'outside' channels connect to adjacent rings. *b*, Side view of three out of the six cavities and channels in the ring. The actual shapes of the cavities and channels are more complex than this diagram indicates.

the parent compounds. Impedance spectroscopy with potassium-coated electrodes (to reduce polarization and the Schottky barrier¹³) over the temperature range 80 to 230 K yielded an apparent activation barrier of 0.064 ± 0.002 eV, but, as shown in Fig. 3, a better fit can be obtained with the equation

$$\sigma = \sigma_0 \exp \left[- (T_0/T)^{1/2} \right] \quad (1)$$

in which T_0 is inversely proportional to the localization length. This result, and the low-temperature magnetic susceptibility, suggest that a variable-range hopping mechanism is responsible

for the observed conductivity^{14,15}.

This electride, and the three with published structures⁶⁻⁸, demonstrate the extreme diversity in the structures and properties of electrides. Both $\text{Cs}^+(15\text{C}5)_2 \cdot e^-$ and $\text{Cs}^+(18\text{C}6)_2 \cdot e^-$ have electron-trapping cavities that are strung out like beads on a chain, some 8.5–9 Å apart, connected by narrow but open channels. They have low electronic conductivities ($\sim 10^{-10} \Omega^{-1} \text{cm}^{-1}$ at 200 K; ref. 13) and relatively weak antiferromagnetic coupling. By contrast, $\text{K}^+(\text{cryptand} [2.2.2]) \cdot e^-$ has spin-paired electrons in elongated traps⁸, and shows high (but thermally activated)

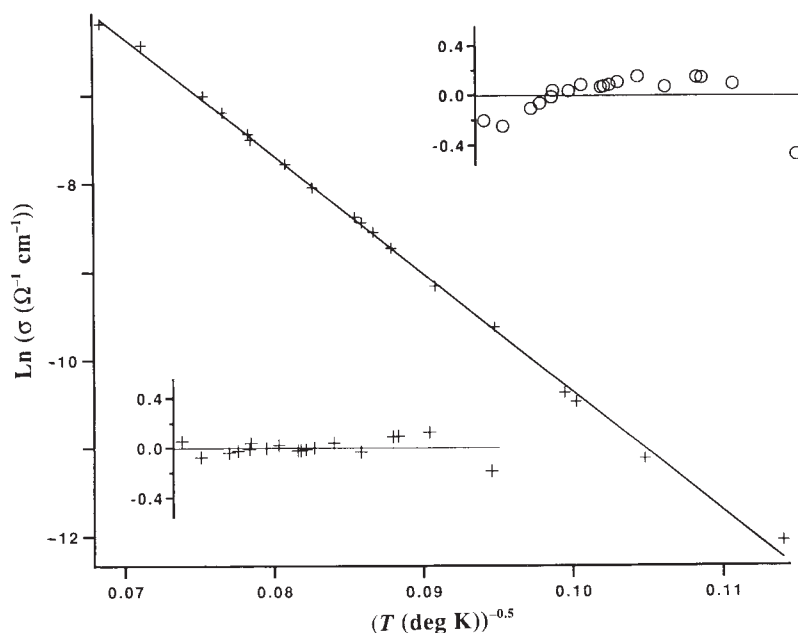


FIG. 3 Least-squares fit of the low-frequency intercept of impedance spectroscopy data by equation (1) (see text). The residuals (calculated – observed) in the upper-right diagram are for a fit of $\ln(\sigma)$ to $1/T$ while those in the lower-left diagram are for a fit of $\ln(\sigma)$ to $1/T^{0.5}$. The least-squares fit by this equation yielded only random deviations with $\sigma_0 = 22.2 \pm 0.7 \Omega^{-1} \text{cm}^{-1}$ and $T_0 = (1.80 \pm 0.02) \times 10^4$ K.

conductivity ($\sim 10 \Omega^{-1} \text{ cm}^{-1}$ at 200 K; ref. 13) and strong anti-ferromagnetic pair-wise coupling⁸. □

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SUPPLEMENTARY INFORMATION. Requests for a complete set of atomic coordinates should be addressed to Mary Sheehan at the London editorial office of *Nature*.

A lower critical ordering transition in a diblock copolymer melt

T. P. Russell*, T. E. Karis*, Y. Gallot† & A. M. Mayes‡

* IBM Research Division, Almaden Research Center, 650 Harry Road, San Jose, California 95120-6099, USA

† Institut Charles Sadron, 6 Rue Boussingault, 67083 Strasbourg Cedex, France

‡ Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

DIBLOCK copolymers, comprised of two distinct homopolymers covalently bonded together at one end, undergo a transition on cooling from a state in which the segments of the blocks are homogeneously mixed to one in which they are segregated locally^{1,2}. This microphase separation is driven by the enthalpy of unfavourable interactions between segments. Here we report the microphase separation of a diblock copolymer melt on heating. Similar in nature to the lower critical solution temperature seen in polymer mixtures, this lower critical ordering transition is driven by entropic factors—specifically, by a negative volume change on mixing of the blocks. The transition to the microphase-separated state alters the rheological and mechanical properties of the copolymer markedly, the material gaining a non-zero equilibrium modulus above the ordering transition. This suggests potential technological applications of these copolymer systems as ‘smart’ materials.

The microphase separation of block copolymers has been of scientific and technological interest for many years^{1,2} because of the increasing use of diblock copolymers as surfactants, impact modifiers and adhesives. Simple homopolymer mixtures phase-separate macroscopically; however, the linking of the two blocks restricts the size scale of the phase separation to the polymer chain dimensions, typically tens of nanometres. In the ordered or microphase-separated state the relative lengths of the blocks dictate the morphology. Spherical, cylindrical, lamellar and bicontinuous morphologies are predicted^{3,8} and have been observed^{9,15}. Interactions between segments, junction point localization to the interface and chain stretching at the interface determine the length scale of the microphase-separated morphol-

ogy. Generally, diblock copolymers undergo a disorder-to-order transition with decreasing temperature¹⁶ which can also be termed an upper critical ordering transition (UCOT). This transition results from an increase in the interaction energy between the segments with decreasing temperature, as quantified by the Flory–Huggins segmental interaction parameter, χ . For most monomer pairs χ is positive, so that increasing the temperature weakens the repulsion between unlike monomers. The nature of the UCOT has been explored experimentally^{17–21} and has been explained by theories that assume incompressibility of the copolymer^{16,22}. Our new findings show that the phase behaviour of diblock copolymer melts is far richer than was previously thought. The discovery of a diblock copolymer that undergoes microphase separation, or ordering, upon heating reveals an unexplored realm in the phase diagram of diblock copolymers.

Symmetric diblock copolymers of perdeuterated polystyrene and poly(*n*-butylmethacrylate), denoted P(d-S-*b*-*n*BMA), were synthesized anionically yielding narrow molecular mass distributions. Although deuteration of the styrene block may alter the observed transition temperatures²³, it is not of significance to the studies presented here. The characteristics of the copolymers are given in Table 1. The weight-average molecular mass of the copolymers ranged from 26×10^3 (26K) to 170×10^3 (170K). For all the copolymers studied, $\sim 50\%$ of the segments were styrene units and so a lamellar microphase-separated morphology is expected as the low-temperature ordered phase. X-ray scattering measurements were performed at the Stanford Synchrotron Radiation Laboratory using monochromatic X-rays with a wavelength λ of 0.149 nm, as described elsewhere²⁴. The beam from the storage ring impinged on the sample in a heating cell that was purged with nitrogen to prevent thermal oxidative degradation. The absence of degradation was confirmed by size-exclusion chromatography measurements before and after the thermal treatments. Samples were compression-moulded at 160 °C into 1-mm sheets.

Figure 1a shows the temperature dependence of the small-angle X-ray scattering profiles from a 68K P(d-S-*b*-*n*BMA) copolymer as a function of the scattering wavevector, $Q(Q = (4\pi/\lambda) \sin \theta$ where θ is the scattering angle), obtained at a heating rate of 5 °C min⁻¹. From 25 to 140 °C the scattering profiles monotonically decrease with Q . For a disordered diblock copolymer, a weak maximum in the scattering is expected due to restricted density fluctuations. This is not observed because the electron density difference between the styrene and *n*-butyl methacrylate segments is small. With further heating a weak maximum emerges at $Q \approx 0.239 \text{ nm}^{-1}$ which corresponds to a Bragg spacing of 26.2 nm. Subsequent cooling shows that the process is reversible, with a loss of this maximum and a return to the monotonically decreasing scattering profile. These data suggest that the copolymer is approaching an ordering transition with increasing temperature. This is the opposite of what has been observed previously and expected from systems exhibiting a monotonic decrease in χ with temperature.

Measurements on the 99K copolymer are shown in Fig. 1b. At temperatures less than ~ 60 °C the copolymer is ordered, as shown by the strong maximum at $Q \approx 0.185 \text{ nm}^{-1}$. This maximum arises from ordered lamellar microphases with a period of 34 nm. The higher-order reflections indicate that the lamellar microphases are well developed. Increasing temperature causes a reduction in the scattering and at ~ 80 °C all vestiges of an ordered morphology are lost. These data are consistent with the classical order-to-disorder transition seen in many diblock copolymers. Increasing the temperature further results in the remarkable growth of a scattering maximum at $Q \approx 0.18 \text{ nm}^{-1}$. This intensifies, and higher-order reflections are again evident. A period of 34.9 nm is found which is nearly identical to that observed at lower temperatures. These results indicate that the 99K copolymer undergoes a second transition from a disordered to an ordered state at elevated temperatures. This transition can be termed the lower critical ordering transition (LCOT). Cooling

the sample causes the maximum to vanish and then reappear at the lower temperatures. For temperatures greater than $\sim 80^\circ\text{C}$, the behaviour is reversible. However, at lower temperatures, the long relaxation times near the glass transition temperature, T_g , of the styrene microphases inhibit the microphase separation process.

The 26K copolymer was always found to be phase-mixed. Regardless of the temperature, no scattering maximum was observed. The 170K copolymer, on the other hand, was always microphase-separated. For all temperatures, sharp, intense scattering maxima were observed at $Q \approx 0.108\text{ nm}^{-1}$, corresponding to a period of 58 nm.

A hypothetical phase diagram of the 99K copolymer is presented in Fig. 2a. At low temperatures (point A) the copolymer is microphase-separated. As the temperature is increased, χ decreases and the diblock copolymer phase-mixes (point B). At higher temperatures the copolymer again microphase-separates (point C). Varying the molecular mass of the copolymer causes the phase boundaries to shift as indicated in Fig. 2b. For the 26K copolymer, the UCOT is below T_g and the LCOT is above the decomposition temperature, T_d . Thus, the copolymer is always phase-mixed. The 68K copolymer is found to be phase-mixed at lower temperatures and, consequently, the UCOT is below the T_g . Only the onset of the LCOT is seen at higher temperatures. Therefore, the LCOT must lie near the T_d . In the case of the 99K copolymer, both the UCOT and LCOT are observable. For higher molecular mass copolymers the UCOT and LCOT appear to merge and the phase boundary is shaped like an hour glass. Although this phase diagram is speculative, it should be qualitatively correct. We do not argue the specific details of the diagrams, only indicate that a new region of the phase diagram for diblock copolymer melts has been uncovered.

The LCOT in diblock copolymers must be similar to the lower critical solution temperature, LCST, seen in homopolymer mixtures and in polymer solutions²⁵⁻³⁰. As opposed to the UCOT,

TABLE 1 Characteristics of P(d-S-b-nBMA) block copolymers

Copolymer	$M_{w,S}^*$	$M_{w,C}^\dagger$	$M_{w,C}/M_{n,C}$	$f_{PS}^\ddagger$
26K	16,000	26,000	1.04	0.67
68K	34,500	68,000	1.02	0.57
99K	47,000	99,000	1.04	0.53
170K	93,100	170,000	1.05	0.6

The copolymers are identified in the text by their mass-average molecular mass $M_{w,C}$ in the first column. $M_{n,C}$ is the number average molecular mass of the copolymer.

* The subscript S refers to the polystyrene block.

† The subscript C refers to the entire copolymer.

‡ f_{PS} is the fraction of styrene segments in the copolymer.

where nonfavourable segmental interactions drive the microphase separation of the blocks, the LCOT is driven entropically. There are several possible routes by which entropic contributions may induce the observed transition. In strongly interacting systems, such as the aqueous block copolymer solutions reported by Mortensen and co-workers^{30,31}, enthalpic interactions can be weakened by volume expansion and hence lead to phase separation. In the absence of such strong interactions, as in the P(d-S-b-nBMA) melt studied here, equation-of-state effects or the conformational asymmetry of the block segments can dominate the phase behaviour. At elevated temperatures, the compressibility of the copolymer melt is significant. If the volume change on mixing is negative, microphase separation will be accompanied by a net increase in the volume which increases the entropy of the system²⁸. These factors must be considered to describe accurately the phase transitions and resulting morphologies of the copolymers. Recent mean field analyses for a compressible melt of P(d-S-b-nBMA) predicts UCOT behaviour (ref. 32, A.M.M., T.P.R. and Y.G., manuscript in preparation) and may suggest new systems which will exhibit such transitions.

Further investigation of the phase behaviour of P(d-S-b-

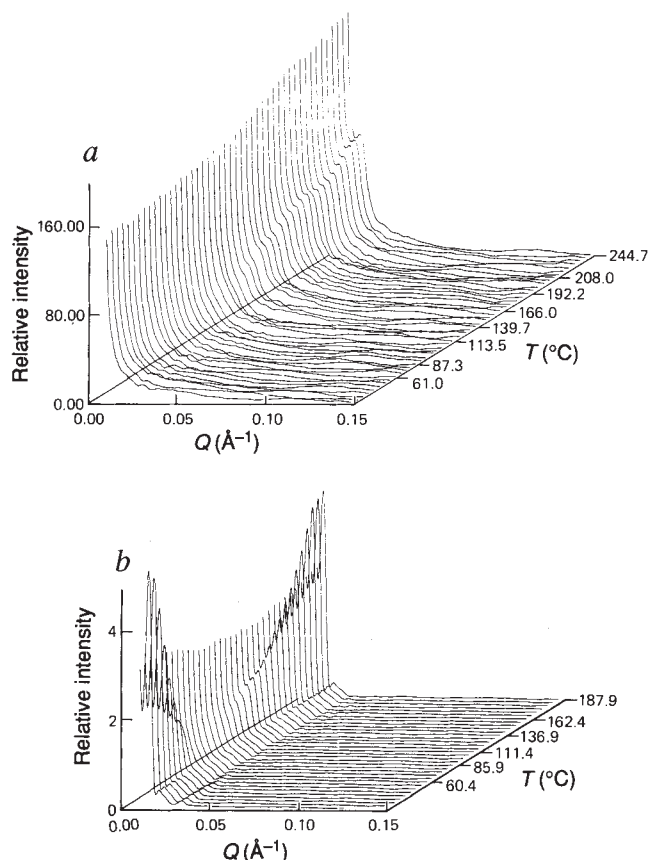


FIG. 1 a, Small-angle X-ray scattering profiles for 68K P(d-S-b-nBMA) as a function of the scattering wavevector, Q , obtained at a heating rate of 5°C min^{-1} . Note the emergence of the scattering maximum at elevated temperatures arising from concentration fluctuations as the lower critical ordering transition (LCOT) is approached. b, Small-angle X-ray scattering profiles for 99K P(d-S-b-nBMA) as a function of Q , obtained at a heating rate of 5°C min^{-1} . Note the loss of the scattering maximum as the copolymer undergoes the classic upper critical ordering transition (UCOT) and the emergence of the scattering maximum at more elevated temperatures as the copolymer undergoes the LCOT.

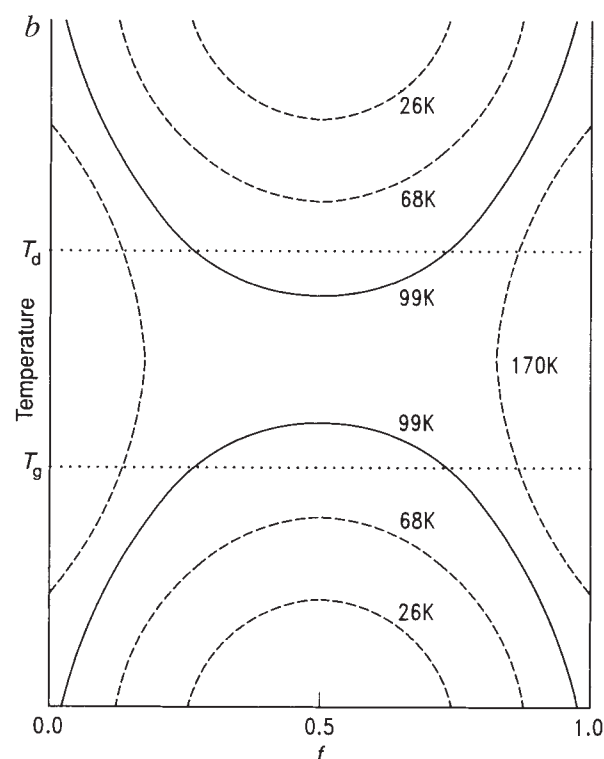
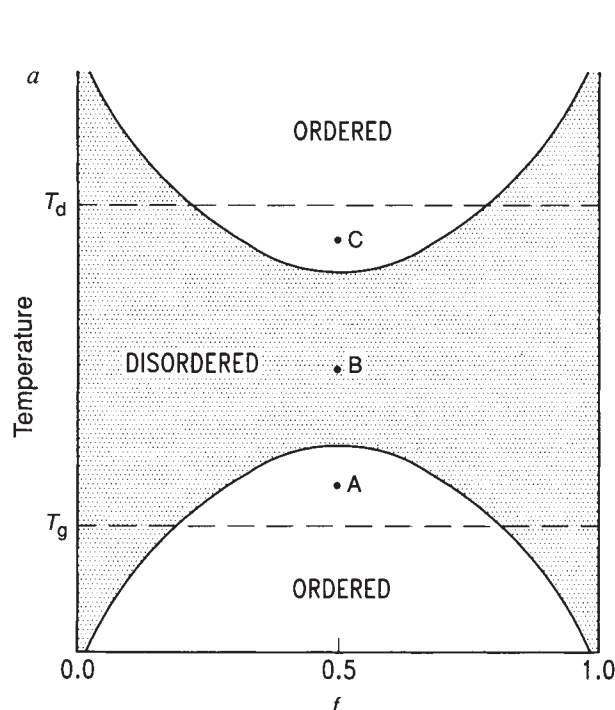


FIG. 2 a, Hypothetical phase diagram of the 99K P(d-S-b-nBMA). f is the fraction of styrene segments in the copolymer, T_d is the decomposition temperature and T_g is the glass transition temperature. b, Hypothetical phase diagram for P(d-S-b-nBMA) for the different molecular masses

indicated. The solid lines indicate that the UCOT and LCOT of the 99K copolymer were observed, whereas the dashed lines are inferred from the data.

nBMA) is currently underway. Preliminary neutron scattering results on the 26K and 68K copolymer clearly show that the scattering increases with increasing temperature, in agreement with the results shown here. In thermo-rheological terms, the 26K, 99K and 170K copolymers appear to be simple in small-amplitude oscillatory shear. The 99K and 170K systems show evidence of an equilibrium shear modulus, indicating an ordered morphology, whereas this behaviour is clearly absent for the 26K material. For the 68K copolymer, the time-temperature superposition principle breaks down above 200 °C and a change in the power-law exponent of the low-frequency storage shear modulus from 2 to 0.5 is seen. This results from the formation of a network that is mechanically equivalent to a critical gel and is characteristic of the microphase separation of block copolymers^{21,33}. These results are in keeping with the scattering observations reported here. Finally, the LCST and UCST behav-

iour for homopolymer mixtures of d-PS and PnBMA was observed recently by neutron scattering³⁴.

The discovery of the LCOT holds promise for new technological applications of copolymers. Synthetic control of the phase boundary defining the ordered state could yield new high-temperature adhesives and thermoplastic elastomers. Within the 'window' defining the homogeneously mixed state, the copolymer can be processed as a conventional thermoplastic. On increasing the temperature, the onset of the ordered phase results in a more rigid material that behaves mechanically as a crosslinked rubber. By varying the copolymer composition and molecular weight, the temperature range of the homogeneous state can be tailored. A further possibility may be new thermal sensor or switch technologies based on the mechanical, rheological or optical property differences between the isotropic and ordered states. □

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Production and transport of methane in oceanic particulate organic matter

David M. Karl* & Bronte D. Tilbrook†

* School of Ocean and Earth Science and Technology, Department of Oceanography, University of Hawaii, Honolulu, Hawaii 96822, USA

† CSIRO, Division of Oceanography, Hobart, Tasmania 7001, Australia

METHANE is an important component of the global carbon cycle¹ and a potent greenhouse gas^{2,3}. Surface ocean waters are typically supersaturated with dissolved methane relative to atmospheric equilibrium, presumably as a result of *in situ* microbial methane production⁴⁻⁸. Because methanogenic bacteria are strict anaerobes⁹ and surface ocean waters are highly oxygenated, the observation of methane supersaturation has been termed the 'oceanic methane paradox'¹⁰. Although methanogenic bacteria have been isolated from oceanic particulate matter, faecal pellets and zooplankton¹¹⁻¹⁴, no data are available on *in situ* rates of methane formation in these microenvironments. During a series of experiments in the North Pacific ocean, we have identified a previously unrecognized component of the oceanic methane cycle. We find that methane is associated with sinking particles, presumably as a dissolved constituent of the interstitial fluids of particulate biogenic materials, which exchanges with the water column as particles sink. This phenomenon provides a mechanism for the active transport in the water column of an otherwise passive, dissolved species. The particle-to-seawater methane flux that we measure is sufficient to replace all of the methane present in the upper water column in about 50 days and to produce the characteristic methane supersaturations in less than a month. We suggest that particulate production and transport may also be relevant to the redistribution and cycling of other bioreactive compounds in the marine environment.

Experiments were performed at five North Pacific ocean stations during cruises of the Vertical Transport and Exchange (VERTEX) and Asian Dust Inputs to the Oceans (ADIOS) research programmes. Two coastal (both at 35.8° N, 122.6° W), one transition (33.3° N, 139.1° W) and two oceanic (35.1° N, 128.2° W and 26° N, 155° W) sites were occupied during this study. Water samples were collected in 10-litre Niskin bottles using standard CTD-rosette (conductivity-temperature-depth) procedures. Subsamples were analysed for dissolved methane concentrations¹⁵, and for a variety of biogeochemical parameters some of which are presented elsewhere^{16,17}. Sinking particles

were collected using MULTITRAP arrays¹⁸, with replicate cylindrical (length 60 cm, inside diameter 7 cm) sediment traps positioned at five or six discrete depths between 50 and 1,500 m depending on the site. Before deployment, each trap was filled with an autoclaved brine solution consisting of 27.1 g NaCl, 11.4 g MgCl₂·6H₂O and 0.74 g KCl per litre of filtered (0.22 µm) surface sea water to provide a final salinity approximately equal to twice that of the surrounding sea water. The brine is used to minimize diffusive loss of collected solutes and to prevent flushing of the traps during recovery. Replicate trap treatments were prepared with the addition of either buffered formaldehyde (0.1 M final concentration) or HgCl₂ (0.5 mM final concentration) to inhibit bacterial activity. From these experimental treatments we could distinguish between methane that entered the traps in association with the sinking particles (plus preservative) from methane production which might occur in the traps after particle collection (minus preservative). All sediment traps were deployed with 335-µm Nitex screens positioned at the base of the baffles. This effectively eliminated contamination by macrozooplankton which, if present, would have seriously compromised the experimental design and data interpretations. This screening procedure is known to cause an approximately 65% decrease in particle collection efficiency¹⁹ and, obviously, selects against large particles that are more likely to contain diffusion-limited anaerobic microhabitats. Consequently, our data on particle-associated methane in the ocean should be taken as a conservative estimate for total transport by this mechanism. The downward flux of 'particulate' methane at each specific reference level in the ocean (expressed in units of nmol m⁻² d⁻¹) was calculated from data on the methane concentration in the sediment-trap brine solution (nmol), the cross-sectional area of the sediment trap (0.0039 m²) and the deployment period (d).

Results indicate that large amounts of methane accumulate in the sediment traps during deployments in both coastal and oceanic habitats (Table 1). The primary conclusion from these field experiments is that dissolved methane is a common constituent of the interstitial fluids of sinking particles in the ocean and that methane partitions into the trap solution phase following particle collection. Adsorption of methane to, and subsequent desorption from, solid-phase particulate constituents cannot be ruled out as another mechanism of transport but seems less likely. A comparison of preserved (formaldehyde or HgCl₂) with unpreserved control treatments supports our conclusion that methane is associated with the particles when they entered the traps, and is not an artefact of post-collection organic matter decomposition (Table 1).

The observed methane fluxes were largest in the near-surface waters and decreased with increasing water depth (Table 1 and

FIG. 1 Methane fluxes as a function of depth, for coastal/transition regions (a) and oceanic habitats (b). The data shown are for paired sediment traps deployed with or without formaldehyde (0.1 M final concentration) as a preservative. The exact station locations are given in Table 1. Both the magnitude of the methane fluxes and the flux divergence are greater in the oceanic regions.

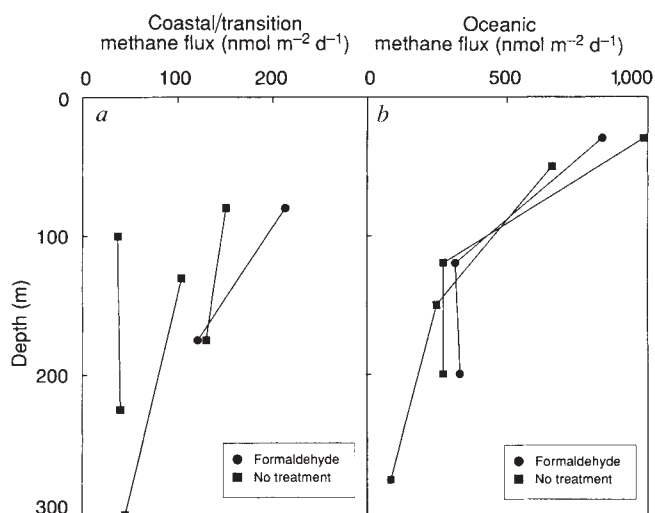


TABLE 1 Representative coastal and oceanic downward 'particulate' methane and particulate carbon fluxes for the north Pacific ocean

Location, date and depth (m)	Deployment period (d)	Preservative added	Trap methane accumulation* (nmol per trap)	'Particulate' methane flux (nmol m ⁻² d ⁻¹)	Particulate carbon flux (mmol C m ⁻² d ⁻¹)	Carbon-specific methane flux (nmol CH ₄ mmol ⁻¹ C)
Coastal (35.8° N, 122.6° W; June 84)	13					
100		None	1.9	36.9	4.1	9
225		None	2.1	40.2	2.1	19
Coastal (35.8° N, 122.6° W; May 85)	12					
80		Formaldehyde	11.2, 8.9†	214	5.7 (±0.6)	38
		HgCl ₂	7.9	169	—	—
		None	7.1 (±1.4)‡	151 (±31)	4.8 (±1.3)	31
175		Formaldehyde	5.6, 5.8	122	3.0 (±0.2)	41
		HgCl ₂	7.0	149	—	—
		None	6.1 (±0.2)	131 (±5)	2.3 (±0.6)	57
Transition (35.1° N, 128.2° W; June 84)	22					
130		None	9.0	105	1.51	70
300		None	3.9	45.6	0.51	89
Oceanic (33.3° N, 139.1° W; June 84)	22					
50		None	56.6	660	—	—
150		None	21.4	250	0.99	252
275		None	7.3	85.2	0.65	131
Oceanic (26° N, 155° W; April 86)	3.1					
30		Formaldehyde	10.1	836	1.19	703
		None	11.9	984	—	—
120		Formaldehyde	3.8	315	1.59	198
		None	3.3	272	—	—
200		Formaldehyde	4.0	331	0.75	441
		None	3.3	271	—	—

* Total methane accumulations were corrected for the time zero system blank, which was determined either by measuring methane concentrations in the trap solutions just before deployment, or by measuring methane concentrations in traps deployed on the same *in situ* array but at much greater depths (600–1,500 m) where the influence of surface methane production and particle-associated transport would be expected to be minimal. The latter system blank was determined to be 5.2 (s.d. ± 1.1; *n* = 14) nmol CH₄ per l of trap solution and did not vary with depth or deployment period.

† Values are from duplicated sediment traps deployed at a common depth.

‡ Mean ± 1 s.d. of the mean for triplicate sediment traps deployed at a common depth.

Fig. 1). These data suggest a euphotic zone particulate methane source, an observation which is consistent with empirical models for the flux of total organic matter^{16,20}. Preservative-containing sediment traps deployed in both coastal and oceanic regions at depths greater than approximately 500 m failed to accumulate methane. These results suggest that the methane production process is probably a surface ocean phenomenon. Bacterial methane oxidizers are known to inhabit the upper water column²¹, and they may be a major sink for the particle-associated methane. Gas exchange with the atmosphere may also be an important sink for methane, especially in areas with large surface ocean supersaturations or deep surface ocean mixed-layers.

Both the magnitude of the observed methane enrichments and the depth-dependent flux patterns varied considerably among the study areas. The estimated methane fluxes in the coastal environments were lower than the open ocean stations, in spite of the much greater flux of particulate carbon inshore (Table 1). The calculated 'carbon-specific methane fluxes' for all regions investigated varied by nearly two orders of magnitude among sites with highest values in the open ocean habitats. Subsequent sediment-trap experiments conducted in surface waters of both coastal (Gerlache strait) and oceanic (Bransfield strait) regions of Antarctica during December 1986–January 1987 (ref. 22), failed to detect any particle-associated methane in spite of an order of magnitude range in particulate carbon fluxes (up to 30 mmol C m⁻² d⁻¹). Therefore, we must conclude that the processes controlling the surface water methane production and flux are geographically variable and probably result from an unique mechanism of particle formation rather than from particle formation *per se*.

As particles sink, the depth-dependent loss of methane is also greater for the oceanic stations (Fig. 1). Methane may be lost from the particle fluids by diffusion, net oxidation or particle disaggregation. From our data we cannot distinguish among these potential sinks, but we currently favour the diffusive loss pathway. If we assume a value of 200 nmol CH₄ mmol⁻¹ C to be a representative estimate of the carbon-specific methane production rate for open ocean habitats (Table 1), then the daily net production of methane in the upper 200 m of the water

column by this mechanism could approach 0.01 mmol m⁻². This estimate must be considered to be conservative because we have not included the likely possibility of particle recycling (that is, production and destruction) within the surface ocean that could also release additional methane. However even this minimum estimate for the production of methane is sufficient to replace all the methane in the upper ocean in approximately 50 days. The methane supersaturations typically observed in the open ocean (~20%, relative to atmospheric equilibrium) could easily be produced in less than one month. Consequently, this hypothesized particle-to-water column flux of methane could be the primary pathway for methane formation in the upper water column.

We hypothesize that methane is formed in zooplankton guts and enters the sinking particle field through the process of faecal pellet formation. The geographical variations seen in total and carbon-specific methane fluxes can be explained either by regional differences in zooplankton species or, more likely, by metabolic differences attributable to food availability. In the food-poor oceanic habitats, the length of time required for gut passage is increased and anaerobic decomposition is more likely to occur than under food-replete grazing conditions²³. The structural integrity of the faecal pellets is likewise affected by feeding rates, favouring the formation of pellets more likely to be mechanically disrupted under periods of food limitation. The ecological predictions of a higher carbon-specific methane content and greater particle-to-water column flux in oligotrophic marine habitats are both supported by our field observations. The decrease in methane production at all locations with increasing water depth is believed to be a reflection of the more general phenomenon of decreased particle production processes¹⁶ and metabolic activity²⁴ observed elsewhere, but this does not rule out the existence of deep production of methane by this or similar mechanisms.

Our present finding that sinking particles can maintain an interstitial fluid composition that may be different from the surrounding sea water suggests that other dissolved compounds and elements may also have pathways of production and transport analogous to those described here for methane. Among these

are other potentially deleterious greenhouse gases such as nitrous oxide and dimethyl sulphide. Furthermore, buoyant rising particles^{25,26} may also effect a flux of dissolved materials to the ocean's surface and thus serve to accelerate the ocean-to-atmosphere transfer of certain gaseous constituents. □

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Declining biodiversity can alter the performance of ecosystems

Shahid Naeem, Lindsey J. Thompson, Sharon P. Lawler, John H. Lawton & Richard M. Woodfin

NERC Centre for Population Biology, Imperial College at Silwood Park, Ascot, Berkshire SL5 7PY, UK

COMMUNITIES of species and their associated biological, chemical and physical processes, collectively known as ecosystems, drive the Earth's biogeochemical processes^{1,2}. Currently most ecosystems are experiencing loss of biodiversity associated with the activities of human expansion^{3–5}, raising the issue of whether the biogeochemical functioning of ecosystems will be impaired by this loss of species^{6–8}. Current ecological knowledge supports a wide range of views on the subject^{9–13}, but empirical tests are few^{9,14–16}. Here we provide evidence from direct experimental manipulation of diversity by over an order of magnitude, using multi-trophic level communities and simultaneous measures of several ecosystem processes, that reduced biodiversity may indeed alter the performance of ecosystems.

The experiment used 14 model, terrestrial microcosms, each 1 m², individually developed, maintained and randomly placed in separate chambers of the Ecotron, a system of controlled environmental chambers designed for such experiments^{17,18}. Air temperature, air flow, relative humidity, water, initial soil conditions, initial densities of organisms, and numbers of trophic levels were the same for all chambers (Table 1).

Plant and animal diversity was manipulated to create low, intermediate, and high diversity microcosms, with 9, 15 and 31 species, structured such that lower-diversity systems contained a subset of higher-diversity species. This manipulation produced a set of microcosms in which lower-diversity communities functionally resembled depauperate descendants of higher-diversity communities that had lost species uniformly across all trophic categories (Table 1 and Fig. 1). This is analogous to what is currently occurring globally in natural ecosystems.

We measured five ecosystem processes: (1) community respiration; (2) decomposition; (3) nutrient retention; (4) plant productivity; and (5) water retention (Table 2). We focused on energetic and chemical ecosystem functions related to the biogeochemical processes of natural ecosystems. We did not

measure community stability, an ecosystem function discussed in other studies^{9,12,13,15,16}.

Statistically, the three levels of diversity represented three treatments: 6, 4 and 4 replicates of high, medium and low diversity treatments were assigned randomly to chambers. Ecosystem functions were measured repeatedly at fixed intervals and analysed using repeated measures analysis of variance (RMANOVA¹⁹). Significance in the highlighted RMANOVA terms (Table 2) supports the hypothesis that different levels of biodiversity are associated with different levels of ecosystem functioning.

The three levels of biodiversity showed significantly different ecosystem performances in the majority of functions (Table 2).

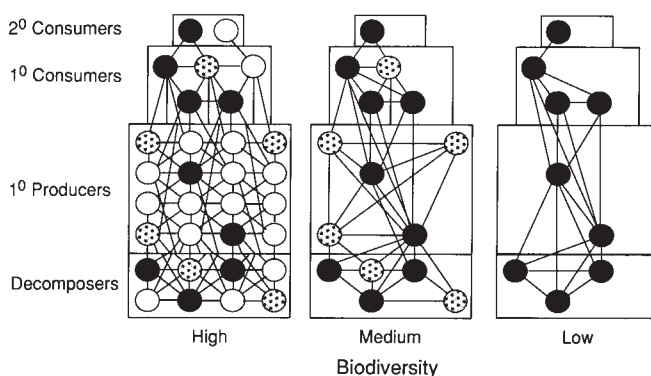


FIG. 1 Community diagrams of the three types of model terrestrial ecosystems developed in the Ecotron. Circles represent species and lines connecting them represent biotic interactions among the species (solid circle, species present in all 3 systems; speckled circle, two systems; open circle, just in the most diverse system). Note that each lower-diversity community is a subset of its higher-diversity counterpart and that all community types have four trophic levels. Primary (1°) producers are plants (self pollinating herbaceous annuals). Primary (1°) consumers are herbivores (molluscs and insects). Secondary (2°) consumers are predators (parasitoids). Decomposers are Collembola and earthworms. See Table 1 for list of species. Larger organisms, such as plants, snails and earthworms, were introduced to achieve equal final densities across all replicates (Table 1) to assure some balance in initial biomass among trophic levels in all replicate ecosystems. Biotic interaction (within level) and trophic (between level) links between species are illustrated; although we have documented trophic links by feeding trials, for clarity we have illustrated only some of the interactions to demonstrate that a loss of species richness is accompanied by a loss in community complexity as well, which is why we use the term biodiversity.

Higher-diversity communities consumed more CO₂ than lower-diversity communities (Fig. 2a). Short-term decomposition rates differed among treatments, but not in any consistent way. Long-term decomposition showed no significant treatment effects. Nutrient retention was different among treatments as measured by total available soil nitrogen, potassium and phosphorus, but with no consistent pattern of variation among treatments. Plant productivity, related in part to community respiration, was higher in the high-diversity ecosystems (Fig. 2b). Water retention was also different among treatments (Table 2), but without any consistent pattern of variation. The positive association between plant diversity and productivity finds some confirmation in the intercropping literature²⁰. It is difficult, however, to compare our results with intercropping experiments because the latter have seldom manipulated more than three species of plants, rarely manipulated other trophic levels, and results have been equivocal²¹.

TABLE 1 Ecotron communities of three differing biodiversities

Community	Plant spp.	Herbivores, predators	Soil fauna
I	<i>Senecio vulgaris</i> <i>Stellaria media</i>	aphid, <i>Myzus ornatus</i>	earthworms, <i>Lumbricus terrestris</i> *
		aphid parasitoid, <i>Aphidius colmanii</i>	Collembola, <i>Megalothorax incertus</i> *
		snail, <i>Helix aspersa</i> slug, <i>Agriolimax reticulata</i>	<i>Folsomia candida</i>
II (+I)	<i>Chenopodium album</i> <i>Spergula arvensis</i> <i>Cardamine hirsuta</i>	aphid, <i>Brevicoryne brassicae</i>	Collembola, <i>Sphaeridia</i> c.f. <i>pumilus</i> <i>Protaphorura</i> c.f. <i>armata</i>
III (+I + II)	<i>Aphanes arvensis</i> <i>Arabidopsis thaliana</i> <i>Capsella bursa-pastoris</i> <i>Conyza canadensis</i> <i>Lamium purpureum</i> <i>Poa annua</i> <i>Sinapis arvensis</i> <i>Sonchus oleraceus</i> <i>Tripleurospermum inodorum</i> <i>Veronica arvensis</i> <i>Veronica persica</i>	white fly, <i>Trialeurodes vaporariorum</i>	Collembola, <i>Proisotoma minuta</i> <i>Pseudosineela alba</i> *
		white fly parasitoid, <i>Encarsia formosa</i>	<i>Mesaphorura machrochaeta</i> *

This lists the nested sets of species in three types of communities used in this experiment. Each set of species is included in the set beneath it. For example, all communities contain the basal species *Senecio vulgaris* but only community III contains *Lamium purpureum*. Seeds were planted on day 1 (23 April 1993). Initial densities of seedlings were held at 40 individuals per plant species in type I, 16 individuals per plant species in type II and 5 individuals per plant species in type III. Initial densities of earthworms were 49 on day 26. Initial Collembola densities were >30, introduced on day 26 with a second introduction on day 111. Initial snail and slug densities were 4 and 5, respectively, introduced on day 69. Additional snails were added each month to bring the final density to 20. Initial densities of aphids, *Myzus ornatus* and *Brevicoryne brassicae*, were 50 and 30, respectively, introduced on day 132, though *M. ornatus* began as a contaminant at an unknown earlier date. Initial white fly densities were 50, added on day 145. Total initial parasitoid (*Aphidius*) densities were 15, established in three introductions of 5, started on day 169. Initial white fly parasitoid densities were 5, introduced with 30 additional white flies on day 165. The experiment ended on day 206. All plant species flowered by day 206. All animals reproduced successfully except those marked with an asterisk. Average environmental conditions for the experiment were: photoperiod, 16 h, between 04:30–20:30, with gradual dusk and dawn durations of 1 h; average light intensity at canopy surface (1 m from lights) when lights were on was 300 μm^{-2} ; pot volume, 0.4 m³; initial soil was 0.1 m³ gravel topped with 0.3 m³ of 40:60 sand/Surrey loam mix, 40.83 p.p.m. N, 12.45 p.p.m. P, 10.69 p.p.m. K; temperature varied smoothly between maximum of 20 °C day and minimum of 12 °C night; relative humidity varied smoothly between maximum of 70% after watering and minimum of 58% before onset of next watering cycle.

The effects of plant community architecture on light interception²² suggests a mechanistic explanation for the differences observed in productivity and community respiration. We measured plant community architecture by two methods (Table 2, rows 6, 7), with higher-diversity systems showing greater per cent cover (Fig. 2c), and a greater number of contacts with sampling pins distributed among greater numbers of height classes (Fig. 2d). Hence, higher-diversity ecosystems intercepted more light because they had better three-dimensional, space-filling canopies, probably accounting for differences in productivity and community respiration. As all ecosystem functions are affected by energy input, it is also possible that this mechanism indirectly accounts for the differences observed in nutrient, water, and decomposition processes, but direct confirmation is not possible for our data.

Our study demonstrates for the first time under controlled environmental conditions, that loss of biodiversity, in addition to loss of genetic resources, loss of productivity¹⁵, loss of ecosystem buffering against ecological perturbation^{9,15,16}, and loss of aesthetic and commercially valuable resources, may also alter or impair the services that ecosystems provide³. However, different ecosystem processes responded differently to loss of biodiversity, providing some support for several current hypotheses^{3,9–11}. To

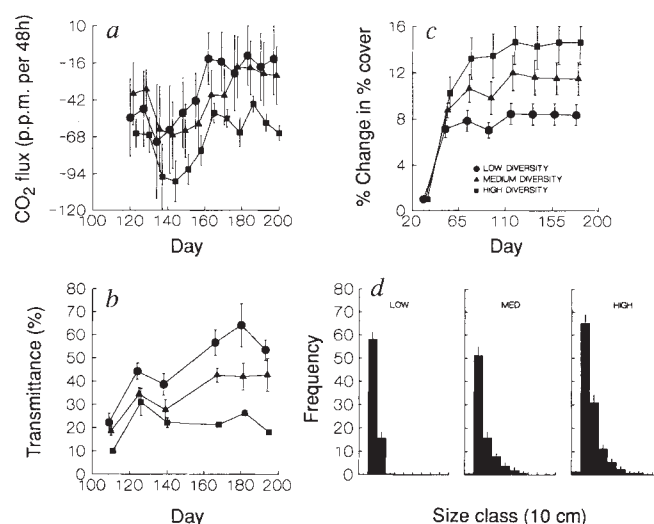


FIG. 2 a, Community respiration (mean $\pm$ 1 s.e.) as measured by CO₂ flux levels for each treatment. Note that the high-diversity ecosystems fixed more CO₂ (flux more negative) for the majority of the time. Measures are flux, in p.p.m. CO₂, over a 48 h period on weekends (no one entering the chamber), with an air flow of 0.25 m³ 50 s⁻¹ per chamber. Cross flow fans within chambers caused turbulent airflow over chamber. Note that differences in fluxes between treatments were small, partly due to the small size of our microcosms; it is therefore difficult to predict the equivalent response of larger systems without further experimentation. b, Plant productivity as measured by per cent transmittance of photosynthetically active radiation. Productivity is correlated with the inverse of per cent transmittance²². Shown are means ($\pm$ 1 s.e.) of per cent transmittance for each treatment. Note that the high-diversity treatment was, from day 107, consistently more productive (lower per cent transmittance) than the low or medium diversity treatments. c, Per cent change in per cent vegetation cover (mean $\pm$ 1 s.e.) from initial conditions as determined by analysis of video images of canopies. ●, Low diversity; ▲, medium diversity; ■, high diversity. Day 1 was 23 April 1993. d, Canopy architectural complexity as measured by pin sampling. Shown are frequency diagrams for encounters of vegetation with vertical pins (24 per pot on a 6 by 4 grid). For clarity, we show only the sixth sample of the seven available, but a similar pattern is observed for other samples (see Table 2 for statistical analyses of all data).

TABLE 2 Ecosystem processes (1–5) and vegetation structure (6, 7) monitored in communities, and repeated measures analysis of variance of results

Ecosystem function		Method	Statistical analyses Term	DF	F	P
1.	Community respiration	Infrared gas analysis of atmospheric input and output for CO ₂ flux, partially detrended for block effect	AMONG*	2, 8	4.5	< 0.05
			WITHIN	11, 88	0.2	0.99 (NS)
			INTERACT	22, 88	2.7	< 0.001
2.	Decomposition short-term surface litter	Change in weight of hay† sealed in inert bags and placed on surface for 6 weeks	AMONG	2, 11	5.4	< 0.05
			WITHIN	1, 11	17.5	<0.01
			INTERACT	2, 11	2.9	0.09 (NS)
	long-term, below ground, wood	Change in weight of wood‡ sticks buried in soil at beginning of experiment	AMONG	2, 11	0.6	0.58 (NS)
			WITHIN	3, 33	145.2	<0.001
			INTERACT	6, 33	0.8	0.76 (NS)
3.	Nutrient retention available nitrogen	Change in soil concentrations	AMONG	2, 25	2.6	0.09 (NS)
			WITHIN	7, 175	65.4	<0.001
			INTERACT	14,175	6.2	< 0.001
	available phosphorus		AMONG	2, 25	5.2	< 0.05
			WITHIN	7, 175	129.6	<0.001
			INTERACT	14, 175	4.3	< 0.001
	available potassium		AMONG	2, 25	9.9	< 0.01
			WITHIN	7, 175	40.1	<0.001
			INTERACT	14,175	3.9	< 0.001
4.	Productivity (plants)	Inverse of % light§ (wavelength, 44–700 nm) transmittance ²⁸ , arcsine-square-root transformed	AMONG	2, 11	38.1	< 0.001
			WITHIN	6, 66	402.5	<0.001
			INTERACT	12, 66	15.1	< 0.001
5.	Water retention	Rate of water outflow	AMONG*	2, 7	2.0	0.21 (NS)
			WITHIN	10, 70	8.1	<0.001
			INTERACT	20, 70	1.8	< 0.05
Vegetation structure						
6.	Per cent vegetative cover (arcsine-square root transformed)	Analysis of video images made every three weeks from day 31 to day 199*	DIVERSITY	2, 11	159.6	< 0.001
			WITHIN	7, 77	5,929.2	<0.001
			WITHIN × DIVERSITY	14, 77	65.5	< 0.001
7.	Pin encounters with vegetation	Pin sampling of 24 positions from ceiling to soil surface in 10-cm increments. All contacts by pin with plants were recorded every three weeks from day 60 to 186	DIVERSITY	2	57.6	< 0.001
			HEIGHT	10	1,273.0	< 0.001
			DIVERSITY × HEIGHT	20	11.6	< 0.001
			ERROR	121		
			WITHIN	6	12.2	<0.001
			WITHIN × DIVERSITY	12	7.4	< 0.001
			WITHIN × HEIGHT	60	9.4	< 0.001
			WITHIN × DIVERSITY × HEIGHT	120	3.4	< 0.001
			ERROR	726		

In analyses 1–5: AMONG, among treatment groups; DF, degrees of freedom associated with F; INTERACT, interaction between within group temporal response and treatment; P, significance probability with critical level set at 0.05; NS, not significant ($P < 0.05$); WITHIN, within temporal series for groups of replicates. Bold type highlights values that indicate significant biodiversity effects. In analysis 6, video data are grouped by diversity treatment in the RMANOVA. In analysis 7, pin sampling uses two grouping factors, one of diversity treatment and the other of height class, in 10-cm increments, of pin encounters. Bold type highlights significance terms important to hypotheses stated in text. Statistical interactions between factors are indicated (for example, WITHIN × DIVERSITY). DIVERSITY, among diversity treatment groups effects. ERROR, error term in breakdown of sums of squares for RMANOVA. HEIGHT, among height, 10-cm increment, groups. Other terms are as previously stated.

* Analysis adjusted for significant block effects.

† A mixture of grasses in the genera *Agrostis* and *Holcus* with a C:N ratio of 35.5:1.

‡ Wood sticks, 11.5 × 0.9 × 0.2 cm, with a C:N ratio of 311:1.

§ Assumes no significant differences in individual leaf transmittance among plant species, which is true for this study.

|| Day 1, 23 April 1993.

the extent that loss of plant biodiversity in the real world means a reduction in the ability of ecosystems to fix CO₂, we also tentatively conclude that the loss of diversity may reduce the ability of terrestrial ecosystems to absorb anthropogenic CO₂ (refs 23–27). □

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Axonal sprouting accompanies functional reorganization in adult cat striate cortex

Corinna Darian-Smith & Charles D. Gilbert*

The Rockefeller University, 1230 York Avenue, New York, New York 10021–16399, USA

* To whom correspondence should be addressed

REMOVAL of sensory input from a focal region of adult neocortex can lead to a large reorganization of cortical topography within the deprived area during subsequent months^{1–9}. Although this form of functional recovery is now well documented across several sensory systems, the underlying cellular mechanisms remain elusive. Weeks after binocular retinal lesions silence a corresponding portion of striate cortex in the adult cat, this cortex again becomes responsive, this time to retinal loci immediately outside the scotoma. Earlier findings showed a lack of reorganization in the lateral geniculate nucleus and an inadequate spread of geniculocortical afferents to account for the cortical reorganization, suggesting the involvement of intrinsic cortical connections^{4,10}. We investigated the possibility that intracortical axonal sprouting mediates long-term reorganization of cortical functional architecture. The anterograde label biocytin was used to compare the density of lateral projections into reorganized and non-deprived cortex. We report here that structural changes in the form of axonal sprouting of long-range laterally projecting neurons accompany topographic remodelling of the visual cortex.

To investigate the presence of sprouting of laterally projecting cortical neurons in functionally reorganized cortex, a comparison was made between axon fibres within normal and reorganized cortex. Receptive field (RF) maps were first documented before, immediately following and 3–9 months after making retinal lesions (Fig. 1 and Table 1). The cortical reorganization observed over this time period extended over an area 7–8 mm in diameter (Fig. 1). Because we were able to conclude from previous work (refs 4, 10 and C.D.-S. and C.D.G., manuscript in preparation) that changes occur largely at the cortical level, the present study focused on intracortical circuitry, particularly the horizontally projecting plexus of cortical neurons^{11–13}. These connections extend laterally for 6–8 mm, and are thought to play a modulatory role in integrating visual information across neighbouring receptive fields. Although the horizontal connections can exhibit use-dependent changes in synaptic weights¹⁴, these changes can occur within seconds and minutes. The reorganization described here, however, takes shape over several months. Our findings indicate these connections may provide the substrate through which visual information is transferred from normal to deprived cortical areas, and through which cortical circuitry is functionally and structurally redefined.

Intrinsic axonal projections were examined by placing extracellular injections of biocytin into cortex just outside the original boundary of the deprived region (cortical scotoma). This allowed labelled axons to be followed laterally from the same injection sites many millimetres into both normal and reorganized cortex. In this instance 'normal' refers to cortical areas representing unlesioned parts of retina, but because there could be distant effects in these areas as well, we also included controls in unlesioned animals. Three-dimensional reconstructions of axons (Fig. 2) projecting through layer III into reorganized cortex revealed a greater exuberance of terminal branching than seen in published examples of horizontally projecting neurons^{11–13}, and suggested that terminal sprouting might accompany functional reorganization. To examine this quantitatively, a comparison of fibre density was made between normal and reorganized cortex as documented physiologically. Fibre density was defined as the sum of the lengths of all fibre fragments found within the sampled volume of cortex. The selected sample regions were positioned at equal distances from the injection strip (see insert in Figs 2 and 3), and extended through the full cortical depth.

Axon fibres were always denser within reorganized than within normal cortex. In three of the four hemispheres analysed, the differences were highly significant (Fig. 3 and Table 1), with fibre densities 57–88% greater in reorganized than in normal cortex. Although in the fourth hemisphere the difference was not statistically significant, the trend was nonetheless similar, and there was a significant increase in density of axonal boutons

TABLE 1 Experimental details of the six hemispheres used

Cat number	Post-lesion survival	Δ Fibre density ($P < 0.05$)	Scotoma size (°)	Distance from injections (mm)	Reorganization extent (mm)	Sample size (mm)
1	8 months	+66%, $P = 0.008$	R = 8 × 16 L = 9 × 17	3.0	R ~ 2.9 L ~ 3.0	3.5 × 0.5
2	14 weeks	+16%, $P = 0.133$	R = 10 × 17 L = 10 × 17	2.0	R ~ 2.5 L ~ 2.5	2.5 × 1.0
3R	8.5 months	+88%, $P = 0.000$	R = 15 × 20 L = 15 × 22	1.0	R ~ 2.0 L ~ 2.2	2.5 × 1.0
3L	8.5 months	+57%, $P = 0.003$	R = 15 × 20 L = 15 × 22	1.0	R ~ 2.0 L ~ 2.2	2.5 × 1.0
4R	Control no lesions	+13%, $P = 0.443$	—	1.3	—	3.0 × 1.0
4L	Control no lesions	+6%, $P = 0.692$	—	1.3	—	3.0 × 1.0

A paired sample *t*-test was used to compare the fibre densities in sample areas (dimensions in furthest right column), through the full sequence of sections cut tangentially from pia matter to white matter. Δ Fibre density is percentage difference between the summed fibre length (across 11–16 sections) in reorganized and normal sample cortex. Analogous sample regions were used for the control hemispheres. R and L, right and left hemispheres in column 1 and left and right eyes in column 4.

in the reorganized cortex of that animal (mean = 28 μ m axon per bouton in reorganized compared with 46 μ m in normal, $P < 0.05$). Preliminary counts indicate that bouton density is at least as great in lesioned animals with longer recovery periods (>8 months) as in unlesioned animals, suggesting that increased fibre density is associated with increased synapse number. Histogram profiles of the horizontal plexus showed a laminar distribution. When reorganized cortex was sampled 2–3 mm from the injection sites, excluding shorter range intrinsic neurons and thalamic afferents, profiles were hourglass in shape (black bars, Fig. 3a), with greatest densities found in supragranular and deep layers. Normal cortex, in comparison, showed bell-shaped profiles of the horizontal plexus (grey bars, Fig. 3a, b; grey and black bars, Fig. 3c), indicating that the greatest change with reorganization occurred within the superficial layers. In all cases, axons never extended further than 4–4.3 mm radially from the injection sites, other than in layer I. Therefore, growth occurred within the normal physical reach of the pre-existing fibre plexus.

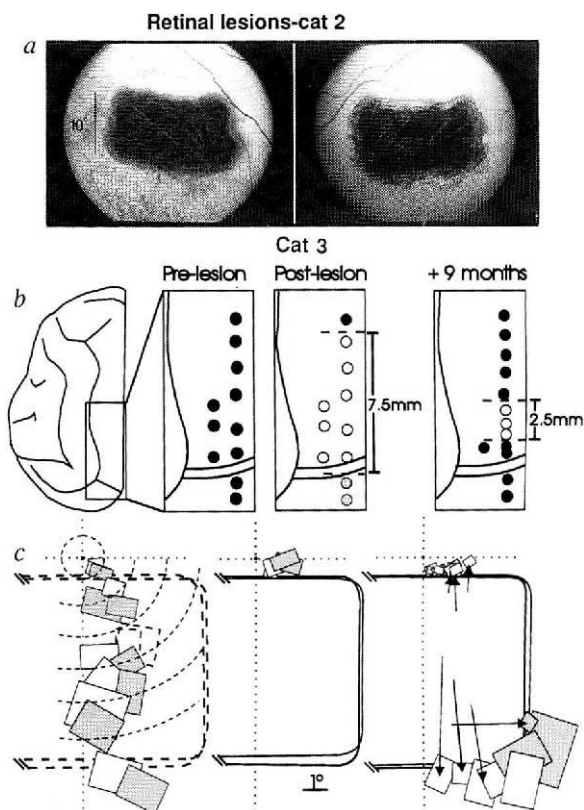
The results indicate that axonal sprouting and presumably synaptic proliferation accompanies topographic reorganization of the striate cortex weeks and months after bilateral retinal lesions have been made. Certain advantages of the extracellular biocytin technique help strengthen the evidence for this. The lesion model allowed us to delineate precisely the boundary between normal and reorganized cortex and to differentiate fibre

patterns between these two areas. Unlesioned cats were used as an additional control to allow for naturally occurring asymmetries in the horizontal projections at different visuotopic eccentricities within the striate cortex. An additional advantage gained by using extracellular injections is that the entire horizontal plexus of axonal fibres projecting into reorganized cortex was labelled, without the sampling bias associated with intracellular labelling studies.

Results from this and earlier work^{3,4,6,10,15} suggest a possible sequence of mechanisms involved in and accompanying different phases of cortical reorganization. In the initial phase⁴, occurring within minutes of making the retinal lesions, there is an expansion of RFs of cells located close to the border of the deprived cortex. This may represent the unmasking of subthreshold excitatory inputs, to a suprathreshold driving control, either by suppressing inhibitory or potentiating excitatory connections. The second phase, marked by the gradual expansion of the representation of perilesion retina into the initially silenced region of cortex, takes place over weeks and months. It is during this extended period that axonal growth and synaptogenesis occur. It may be that these morphological responses/adaptations to sustained sensory loss allow the cortical circuit to adjust to a new level around which rapid synaptic modulation could occur. Importantly, the morphological changes described respect the limits set by the pre-existing fibre plexus, resulting in the enrichment of fibre clusters rather than an apparent extension of fibres

FIG. 1 Topographic reorganization of striate cortex after binocular retinal lesions. RFs were mapped from cells within a series of vertical electrode penetrations in the superficial layers, placed rostrocaudally through the striate cortex in adult cats. The example shown is from cat number 3 (b, c). Maps were made at corresponding sites before, immediately after and months after lesioning the eyes. Cortical vasculature and dural scarring served as fiducial landmarks. In all cats, matching focal retinal lesions were made in the inferior visual hemifield, abutting but outside the area centralis (a, c). The lesions were photographed with a fundus camera (Zeiss FF4, a). Post-mortem histology showed that the pigment epithelium and photoreceptor layer were destroyed by the lesion, leaving the ganglion cell layer and vasculature intact. Retinal lesions in the two eyes were about $10^\circ \times 17^\circ$ (see Table 1), traversed the vertical meridian to involve the two hemifields, and typically overlapped within visual space to an accuracy of 0.5° arc. Immediately after lesions were made, visually driven activity could not be elicited from cells within 7.5 mm of cortex, or 15° of visual space. Nine months later, however, 5 mm of the original cortical scotoma recovered visually driven activity, this time responding to stimuli presented immediately outside the retinal lesions. In visual space, RFs had shifted 6° – 7° from their original positions inside the scotoma, to lie outside it (see arrows c).

METHODS. Retinal lesions, electrophysiological recording and iontophoretic injections of biocytin were made in anaesthetized, paralysed cats⁴. Lesions were made with multiple flashes from a diode laser (Iris Instruments, 800 ms $\times$ 800 mW each). During the terminal experiment and after RFs had been mapped for the final time, a mediolateral strip of 3 biocytin injections (separated by about 1 mm and spanning the full depth of cortex) were placed just behind the original border of the deprived region of cortex, and the animal was perfused one day later. This border was localized according to pial vasculature patterns and dural scarring, recorded in photographs taken during earlier experiments. It was then possible to compare the densities of axons projecting from normal cortex into both reorganized and normal cortex. Axon fibres were reconstructed within sample regions at equal distances either side of the same injections (Fig. 3), and for sequential sections cut tangentially through the full thickness of cortex. The use of three injections of 300–800 μ m diameter in each hemisphere and the reconstruction of fibre fragments within large sample areas lessened artefactual bias towards orientation or ocular dominance columns³¹. Only hemispheres with injection sites confined to grey matter were used. Injections of 4% Biocytin (Sigma) in saline were delivered iontophoretically (15 μ m tip diam) using 2.5–3.5 μ A positive current pulsed at 8-s intervals for 3–4 min. Two injections were made within each vertical penetration. Animals were perfused, the tissue was cut tangentially to the dorsal surface at 70–80 μ m, and then processed using ABC reagent (Vector) and DAB



as the chromagen. Retrogradely labelled neurons were rarely observed, and were pale in appearance when compared to anterogradely labelled cells³². Only anterogradely labelled axons were then reconstructed from sequential sections (Eutectics 3D Tracing System). Total length of fibre fragments and number of axonal boutons were documented within the reorganized and normal sample regions of each section, and paired according to depth from the pial surface. A dependent sample t-test was used for all calculations, for the full set of sections, reconstructed from pial surface to white matter. Results are listed in Table 1 and plotted in Fig. 3.

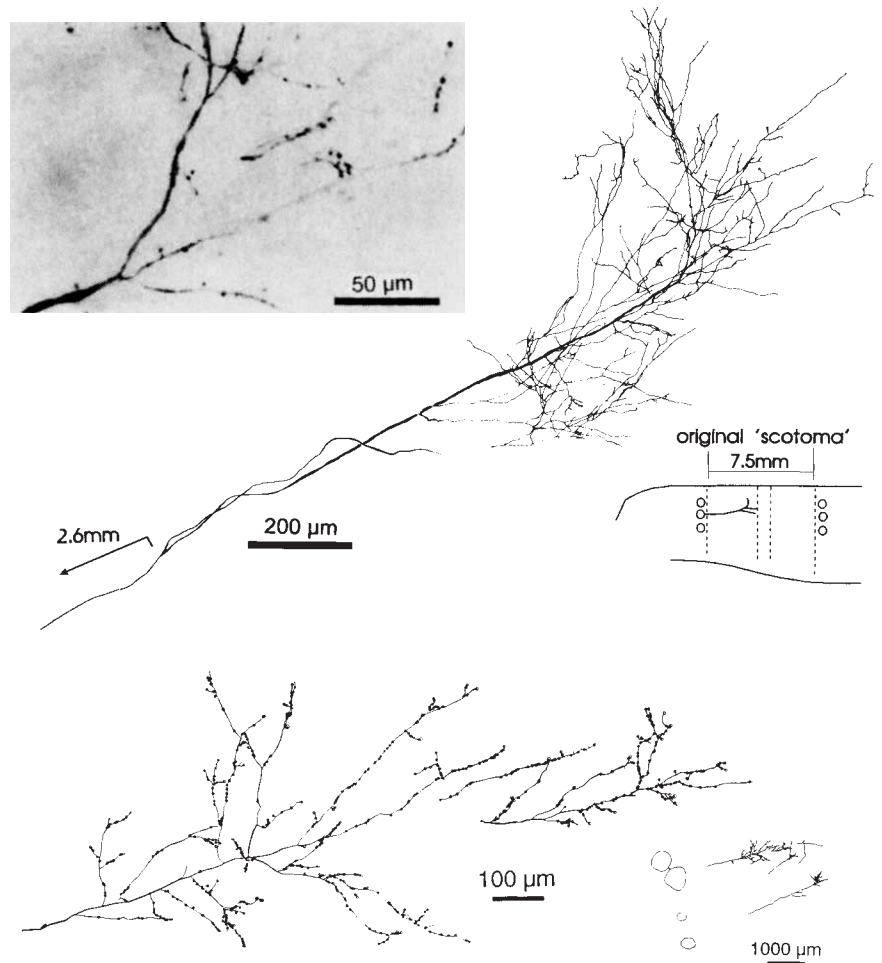
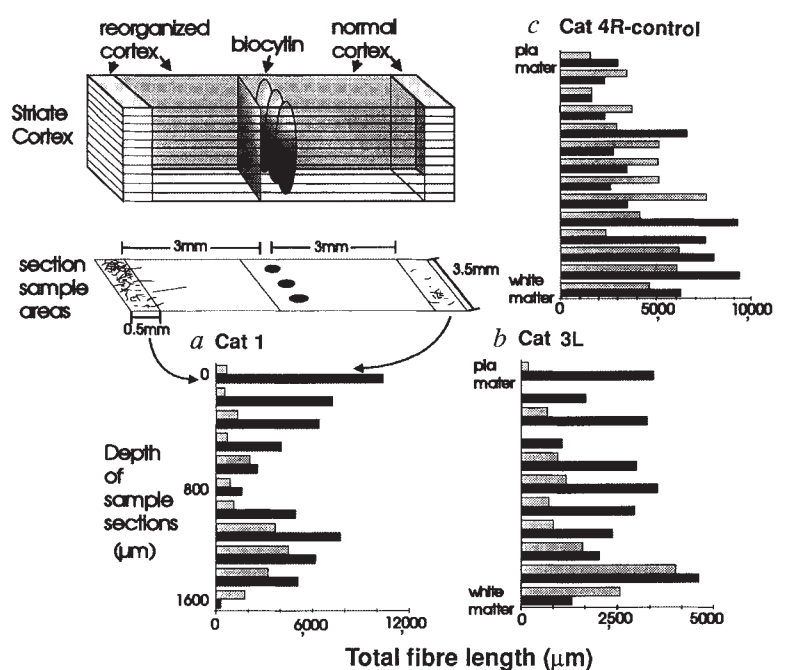


FIG. 2 Reconstructions of axon terminal tufts projecting from injections placed outside the original border of deprived cortex, into the reorganized cortical area. Both axons were reconstructed from layer III in the right hemisphere of cat number 1 (not used in the present analysis). Primary axons could be traced within two adjacent tangential sections for several mm back to injection sites but not to cell somata. The physical extent and richness of arborization contrasted with previously published accounts of long-range laterally projecting neurons in striate cortex¹¹⁻¹³.

FIG. 3 Schematic of experimental model, showing placement of biocytin injections relative to cortical scotoma; and typical fibre density histogram profile for two lesioned (a, b) and one control (c) cat. Total fibre length (fibre density) was calculated as the length of fibre fragments summed within sample areas positioned at equal and constant distances (within any one hemisphere) either side of injection strips. Vertical abscissa (0–1,600 µm) indicates standardized depth of sections from pia to white matter; differences in the scale of the horizontal abscissae reflect differences in the size of injections within each hemisphere and thus the numbers of fibres labelled. Grey and black bars refer to analogous sample locations within striate cortex, in both reorganized and control hemispheres. Details of lesion dimensions, sample sizes and duration of recovery can be found in Table 1. Although axon fibre densities were significantly greater in reorganized cortex than in normal cortex (compare black with grey in a, b), similar differences were not observed within the control hemisphere (compare black with grey in c). Histogram profiles of the horizontal plexus showed a laminar distribution. In the reorganized cortex of lesioned cats (a, b), the greatest changes occurred consistently within the superficial layers. In cats where cortex was sampled 2 or 3 mm distant to the injection sites (for example, a), the profile was hourglass in shape, with lowest fibre densities occurring within layer 4, where thalamic afferents predominantly terminate. Density profiles for normal cortex (grey in a, b; grey and black in c) in both reorganized and control hemispheres were typically bell-shaped with greatest densities found within infragranular layers.



beyond the normal. This observation implies an upper limit for the physical extent of cortical reorganization and is consistent with findings in other sensory systems^{8,16,17}.

Synaptogenesis and sprouting have previously been affiliated with functional remodelling of Aplysia¹⁸, and in a variety of structures in the adult mammalian central nervous system, including the hippocampal formation¹⁹, ascending and descending pathways of the spinal cord^{20–24} (M. Galea and I. Darian-Smith, manuscript in preparation), in the brainstem^{22,25}, thalamus²⁶, and most recently in motor cortex after cerebellar lesions²⁷ and electrical stimulation^{28,29}. The findings presented here may be distinguished by a combination of features. Axonal sprouting is occurring within adult sensory neocortex, is directly

related to functional changes, and is occurring in response to lesions made at the sensory periphery, without cortical deafferentation^{4,10} or synaptic vacancies being left by retracted connections. Although these results show sprouting of intrinsic connections as an important element in cortical reorganization, they do not exclude potential additional influences by distant cortical and subcortical areas. It is tempting to suggest that sprouting and synaptogenesis accounts for some of the topographic reorganization reported in other sensory systems, following peripheral manipulations and even training^{1–9,29,30}. Our findings raise the possibility that terminal sprouting and synaptic proliferation represents part of the normal response of the adult cortex to extended modification of sensory experience. □

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A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation

Z. A. Bortolotto, Z. I. Bashir, C. H. Davies & G. L. Collingridge

Department of Pharmacology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK

PHARMACOLOGICAL studies of long-term potentiation (LTP) in the hippocampus are starting to provide a molecular understanding of synaptic plastic processes which are believed to be important for learning and memory in vertebrates¹. In the CA1 region of the hippocampus, the synaptic activation of glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype is necessary for the induction of LTP under most experimental conditions^{2,3}. The synaptic activation of metabotropic glutamate receptors (mGluRs) is also needed for the induction of LTP^{4,5}. We now show that the role of mGluRs in the induction of LTP is fundamentally different from that of NMDA receptors. NMDA receptors initiate a molecular event that needs to be triggered each time a tetanus is delivered to induce LTP. In contrast, mGluRs activate a molecular switch which then negates the need for mGluR stimulation during the induction of LTP. This mGluR-activated switch is input-specific and can be turned off by a train of low-frequency stimulation. The molecular switch is a new feature of LTP which has fundamental consequences for our understanding of synaptic plastic mechanisms.

The specific mGluR antagonist ($\pm$)- α -methyl-4-carboxyphenylglycine (($\pm$)MCPG)⁶ blocks the induction of LTP, but

not short-term potentiation (STP), in the Schaffer collateral-commissural pathway^{4,5}. The activity of MCPG as a mGluR antagonist resides solely in the (+) isomer⁷. Consistent with the block by MCPG being due to antagonism at these receptors, (+)MCPG, but not (–)MCPG, blocked the induction of LTP when tested sequentially on three slices (data not shown). We therefore used (+)MCPG throughout. We tested 200 μ M MCPG on 39 slices that had not received any other treatments (naïve slices). In 33 of these slices, the induction of LTP was blocked (the response measured 60 min following a tetanus was $101 \pm 1\%$ control; range 95–108%). Thus, tetanic stimulation in the presence of MCPG induced STP but not LTP and after washout of MCPG tetanic stimulation induced LTP. In 6 slices, however, the induction of LTP was not prevented by MCPG (the response measured 60 min following a tetanus was $133 \pm 2\%$ control; range 130–142%; data not shown).

mGluRs can initiate transient signals, such as the formation of inositol phosphates, and sustained signals, such as phosphorylation through the activation of protein kinase C^{8,9}. We reasoned that if mGluRs initiate a persistent change, then mGluRs might not need to be activated during each tetanus. We therefore established that MCPG blocked the induction of LTP; after washout of MCPG we induced LTP and then stimulus-matched the potentiated response to control levels. Finally, we tested the ability of MCPG (200 μ M) to block the induction of further LTP. Invariably MCPG failed to prevent the induction of further LTP ($n=5$; Fig. 1a, b). In case 200 μ M was insufficient to block the induction of further LTP, after LTP had been induced, we repeated the experiment with a fivefold higher concentration of MCPG. In each slice tested, MCPG (1 mM) failed to prevent the induction of further LTP ($n=5$; Fig. 1c).

These experiments show that there is a conditioning effect of an LTP-inducing tetanus which negates the need for the synaptic activation of mGluRs for the induction of further LTP. We have assumed that this conditioning process is initiated by the synaptic activation of mGluRs during the LTP-inducing tetanus.

Three series of experiments were performed to test this idea. In the first, we stimulated mGluRs by application of the specific agonist 1-aminocyclopentane-(1*S*, 3*R*)-dicarboxylate (ACPD). Application of 10 μ M ACPD for 20 min induced slow-onset potentiation, as reported previously¹⁰. In each case, after the baseline had been reset, MCPG did not block the induction of LTP ($n=4$; Fig. 2*a*). In parallel experiments, we delivered low-frequency (900 shocks at 1 Hz) stimulation immediately after the application of ACPD to prevent the development of slow-onset potentiation¹¹. In these experiments MCPG subsequently blocked the induction of LTP ($n=4$; Fig. 2*b*). These results show that stimulation of mGluRs can produce the conditioning and that this effect involves processes that are disrupted by low-frequency stimulation. In the second series of experiments, we activated mGluRs synaptically by delivering tetanic stimulation. This was done in the presence of the specific NMDA-receptor antagonist D-2-amino-5-phosphonopentanoate (AP5; 50 μ M) to block the induction of LTP. Under these conditions, following washout of AP5, MCPG did not block the induction of LTP

($n=4$; Fig. 2*c*). In these experiments a second independent input, which did not receive tetanic stimulation during exposure to AP5, was used. In this input, MCPG blocked the induction of LTP. In the third series of experiments, we blocked the activation of mGluRs during the first (conditioning) tetanus. Thus, we compared the effects of delivering two tetani to each of two inputs; the first (conditioning) tetanus in either the presence or absence of MCPG and the second (test) tetanus in the presence of MCPG. When both the conditioning and test tetani were given in the presence of MCPG, LTP was not induced. In contrast, when the conditioning tetanus was delivered in the absence of MCPG (so that LTP was generated) then MCPG failed to block the induction of further LTP in response to the test tetanus ($n=6$; Fig. 3*a*). Considered together, these experiments show that the conditioning effect provided by a tetanus does not depend upon the induction of LTP but is blocked by an mGluR antagonist and can be mimicked by application of an mGluR agonist. In addition, the experiments illustrated in Figs 2*c* and 3*a* show that the conditioning effect of a tetanus is input-specific.

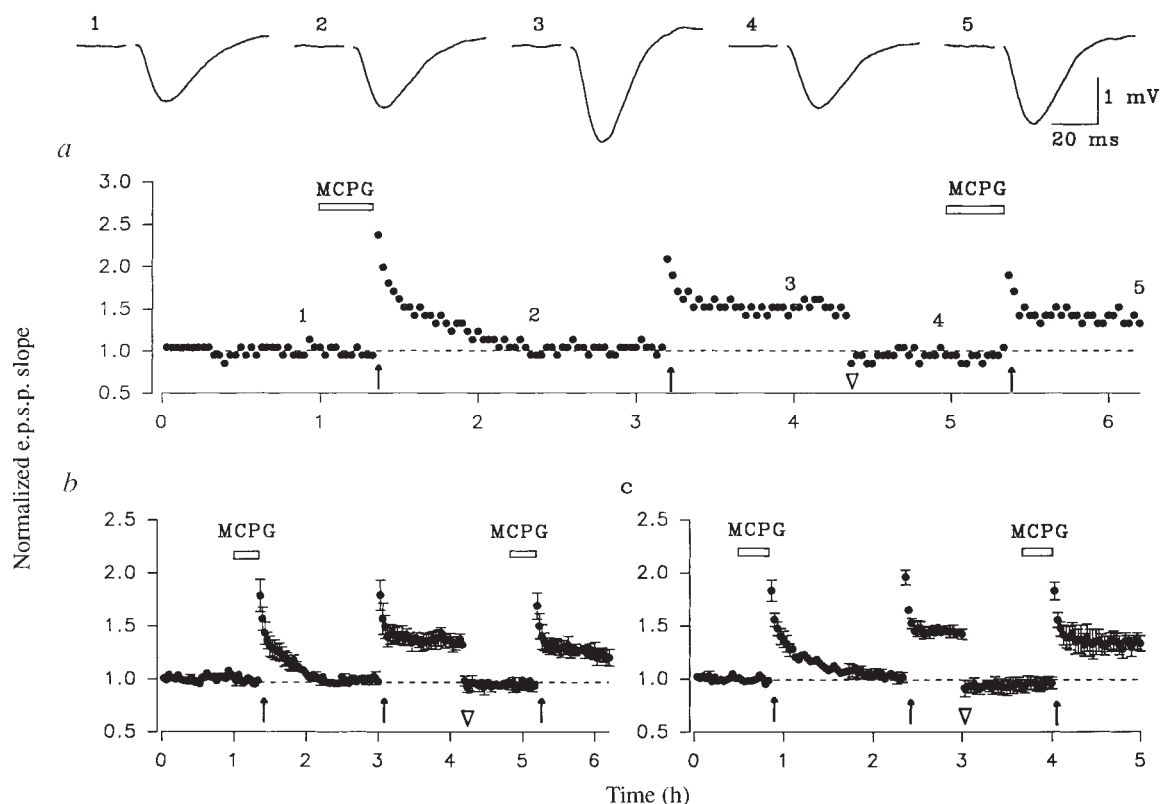


FIG. 1 The prior induction of LTP prevents MCPG from blocking the induction of further LTP. *a*, A single experiment to show the effects of MCPG on the induction of LTP both before and after a slice has been conditioned by the induction of LTP. When the slice was in a naive state MCPG blocked the induction of LTP (but not STP). After washout of MCPG, a second tetanus-induced LTP (MCPG was applied immediately following the tetanus to show that the MCPG-sensitive process occurs during but not after the tetanus). After LTP had been followed for 70 min, the baseline was reset by reducing the stimulus intensity (∇). A third tetanus (the second one delivered in the presence of MCPG) induced further LTP. The synaptic records are averages of 4 successive sweeps of field excitatory postsynaptic potentials (e.p.s.ps) obtained at the times indicated by the numbers. *b*, Pooled data from 5 experiments as in *a*. *c*, Pooled data from 5 experiments as in *a*, except that the third tetanus was delivered in the presence of 1 mM MCPG (also, MCPG was not applied immediately after the second tetanus). METHODS. Experiments were performed at $\sim 30^\circ\text{C}$ on transverse hippocampal slices from 4–6-week-old rats using standard techniques. Extracellular recordings were made from stratum radiatum of area CA1

in response to single-shock stimulation of the Schaffer collateral-commissural pathway. In single input experiments the pathway was stimulated once every 30 s. When two inputs were studied, stimulating electrodes were placed on either side of the recording electrode and each pathway was stimulated once every 60 s, with 30 s separating the stimulation of each input. Independence of the two inputs was established using a paired-pulse protocol. The slope of the rising phase of the field e.p.s.p. was measured between ~ 20 and 80% of peak amplitude. Each point on the graph is the mean value of 4 successive responses. Each figure (except for Figs 1*a* and 4*a*) plots pooled data for each experiment in which a particular protocol was used. The symbols show the mean (± 1 s.e.m., where this is larger than the symbol). (+)MCPG (200 μ M, unless otherwise indicated), (1*S*, 3*R*)-ACPD (10 μ M), D-AP5 (50 μ M) and K-252b (100 nM) were applied by addition to the perfusate for the times indicated by bars above the graphs. A tetanus comprised 100 Hz for 1 s, delivered at the times indicated by arrows. Low-frequency stimulation comprised 900 shocks at 1 or 2 Hz delivered at the time indicated by a bar below the graph.

We next explored the possibility that the conditioning involved activation of a protein kinase. We delivered a conditioning tetanus in the presence of the potent, but reversible, protein kinase inhibitor, K-252b^{10,12}. This agent blocked reversibly the induction of LTP, but not STP ($n=4$; Fig. 3b, input 2), and prevented the conditioning so that MCPG blocked the induction of LTP ($n=4$; Fig. 3b; input 1). To investigate how long the mGluR-mediated conditioning effect lasted, we induced LTP in two inputs and then tested the ability of MCPG to block the induction of further LTP after 3 and 6 h in the two respective inputs. At both times, MCPG did not block the induction of LTP ($n=3$; data not shown). Thus, mGluR-mediated conditioning seems to involve activation of a protein

kinase and the effect does not reverse passively within the time-scale of our *in vitro* experiments.

As low-frequency stimulation prevented the conditioning effect of ACPD, we reasoned that it should also prevent the conditioning effect associated with a tetanus. In naive slices, low-frequency stimulation (900 shocks at 2 Hz) did not induce long-term depression (LTD; $n=16$; Fig. 4a). In contrast, following

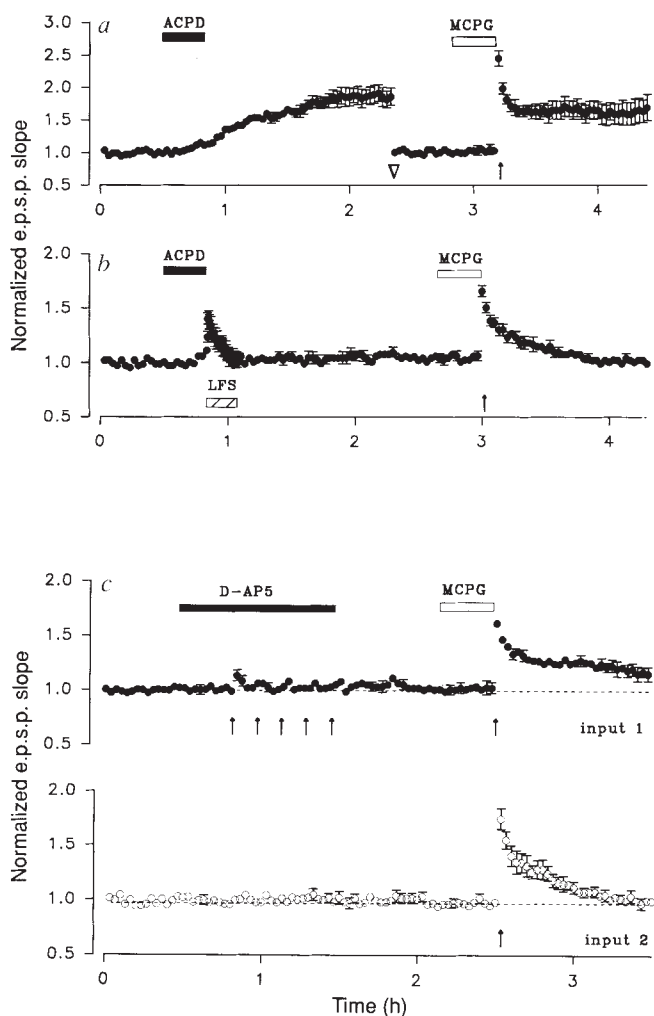


FIG. 2 Activation of mGluRs conditions the pathway so that MCPG fails to block induction of LTP. *a*, Slow-onset potentiation was induced by addition of ACPD. After a plateau potentiation had been reached, the baseline was reset by reducing the stimulus intensity (∇). MCPG then failed to block the induction of LTP. In *b*, slow-onset potentiation was prevented by delivering low-frequency stimulation (LFS) immediately following the application of ACPD, and MCPG blocked the induction of LTP. (The transient potentiation is due to frequency facilitation during the low-frequency stimulation.) *c*, Input 1: tetanic stimulation (5 tetani delivered at 10-min intervals) in the presence of AP5 (50 μ M) induced conditioning but not LTP. Input 2: this input did not receive tetanic stimulation and MCPG blocked the induction of LTP. Note that the conditioning by tetanic stimulation of input 1 did not condition input 2. Each graph plots pooled data from 4 slices.

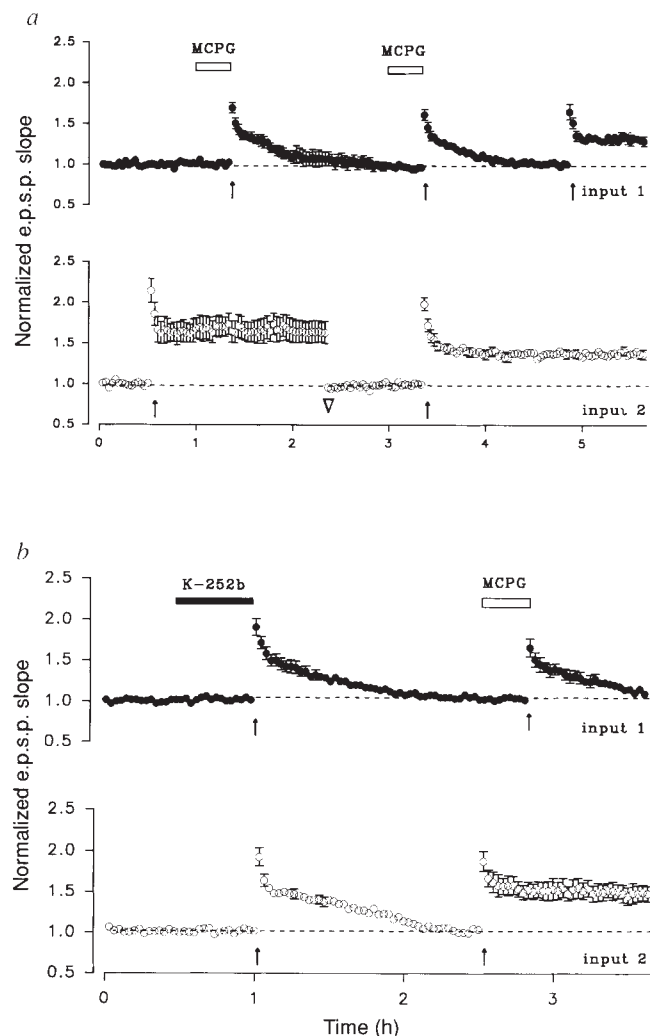
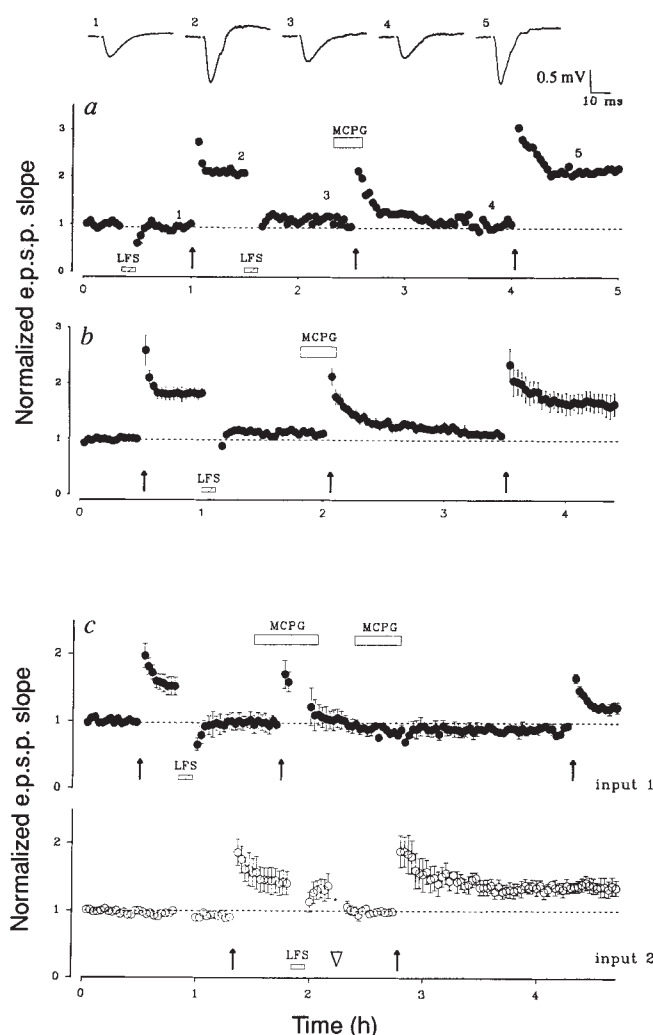


FIG. 3 The conditioning effect of a tetanus is mediated by an MCPG-sensitive, protein-kinase-dependent process. *a*, Pooled data from 6 slices. Input 1: the first (conditioning) tetanus was delivered in the presence of MCPG. This treatment blocked the induction of LTP and the conditioning effect of the tetanus, because a second (test) tetanus delivered in the presence of MCPG also blocked the induction of LTP. Following washout of MCPG, a third tetanus induced LTP. Input 2: the first tetanus (conditioning) was delivered before application of MCPG and resulted in LTP; 110 min later, the baseline was reset by reducing the stimulus intensity (∇). The second (test) tetanus was delivered in the presence of MCPG and induced further LTP. Thus, MCPG prevents the conditioning effect of a tetanus. Note also that the induction of LTP in input 2 did not prevent MCPG from blocking LTP in input 1. *b*, Pooled data from 4 slices. Input 1: K-252b (100 nM) blocked the induction of LTP and the conditioning effect of a tetanus, because MCPG blocked the induction of LTP. In two of these experiments a third tetanus was delivered after washout of MCPG and LTP was induced on both occasions (not shown). Input 2: in this pathway, the second tetanus was delivered immediately before addition of MCPG to show that K-252b had washed out of the system (that is, the block of LTP in input 1 was due to MCPG rather than K-252b).



the induction of LTP low-frequency stimulation led to depotentiation^{13,14}, which almost restored the synaptic response to base-line levels. In each case, following depotentiation, MCPG blocked the induction of LTP ($n=8$; Fig. 4). In contrast, when low-frequency stimulation was delivered in the presence of MCPG to inhibit depotentiation¹⁵, MCPG was unable to block the induction of LTP ($n=3$; Fig. 4c). Thus, low-frequency stimulation provides a means of deconditioning, as well as depotentiating, this synaptic pathway through a mGluR-mediated process.

This study has revealed a new and surprising property of LTP.

FIG. 4 Low-frequency stimulation de-conditions the LTP process. a, A single experiment to show that low frequency stimulation (LFS) did not induce any long-lasting change in synaptic efficiency in naive slices. Following the induction of LTP, however, low-frequency stimulation depotentiated the pathway to almost pre-tetanus levels. MCPG was then able to block LTP reversibly. This experiment was repeated in 3 slices with identical results. b, Pooled data for 6 slices (3 of which were preceded with a period of low-frequency stimulation; results not shown). c, Pooled data for 3 slices. Input 1: this input was treated as in b; input 2: following the induction of LTP, low-frequency stimulation was delivered in the presence of MCPG and depotentiation was prevented. After the base-line was reset (∇), and re-normalized, MCPG failed to block the induction of LTP. During the delivery of low-frequency stimulation to one input, stimulation of the other input was stopped.

Whereas NMDA receptors need to be activated every time to induce LTP^{1,2}, mGluRs need only be activated once. Conceptually, NMDA receptors can be thought of as a necessary trigger, which automatically re-cocks, whereas mGluRs provide a switch that, once activated, stays on. This switch can, however, be reset by low-frequency stimulation.

The precise mechanisms involved in the setting and re-setting of the switch need to be determined. The observation that both MCPG and K-252b prevent conditioning is consistent with the setting involving mGluR-mediated activation of protein kinase C leading to phosphorylation. The observation that MCPG also prevents deconditioning indicates that mGluRs are also involved in resetting of the switch. As low-frequency stimulation generates LTD through the activation of phosphatases¹⁶, it is tempting to speculate that deconditioning involves dephosphorylation. The reason that different stimulation paradigms can both set and reset the switch as a result of the activation of MCPG-sensitive processes could be because different subtypes of mGluRs are preferentially activated according to the frequency of stimulation. Alternatively, additional factors controlled by the frequency of synaptic activation may determine the direction of the setting of the switch. A parallel situation exists in which NMDA-receptor activation can lead to either LTP or LTD, depending on the frequency of stimulation^{1,17,18}.

In most of our experiments the conditioning and the induction of plasticity coincided. But they could be separated because tetanic stimulation in the presence of AP5 was able to condition without inducing LTP. In addition, low-frequency stimulation, delivered thereafter, is able to decondition without incurring LTD (unpublished observations). Furthermore, a single tetanus was sufficient to condition fully a pathway but did not maximally induce LTP, because further LTP was always produced by a second tetanus. Similarly, a single application of ACPD fully conditions a pathway, but does not maximally induce potentiation¹⁰.

In conclusion, mGluRs are involved in the generation of both LTP⁴ and depotentiation¹⁵. With respect to LTP, they are needed for its induction when slices are in a deconditioned state, because they enable a conditioning switch to be set. The mGluR-controlled switch adds a new dimension to our understanding of synaptic plasticity. □

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ity, inhibiting clinical symptoms in the CD4-dependent autoimmune disorder EAE. The level of incidence of EAE (mice with symptoms at any grade) in the untreated group reached 83% by day 19 of the experiment and 71% in the group treated with the control peptide (scrambled rd-mPGPtide), a peptide with an identical amino-acid composition to the rd-mPGPtide, but with a scrambled sequence order (Fig. 3a). By contrast, all groups

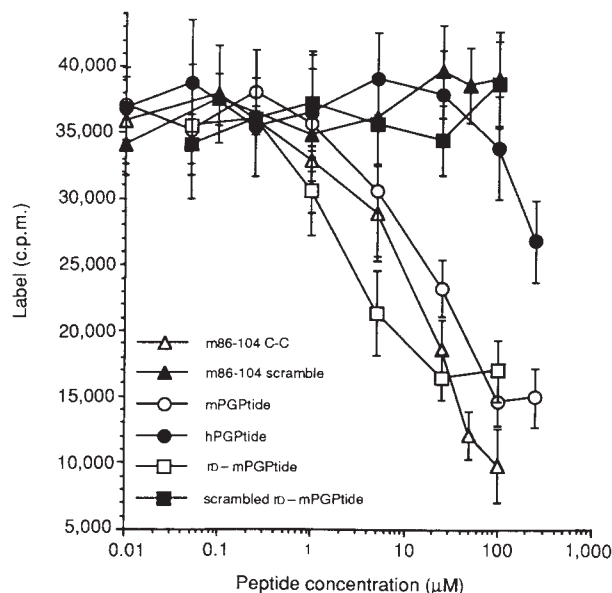
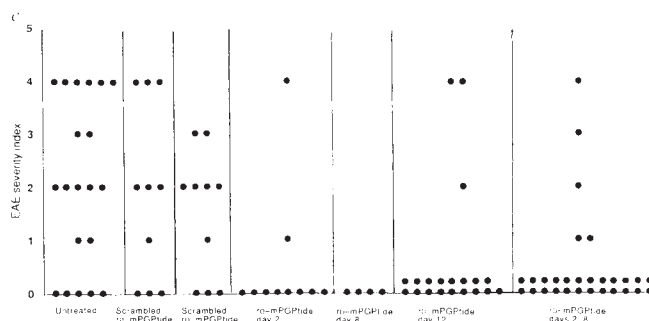
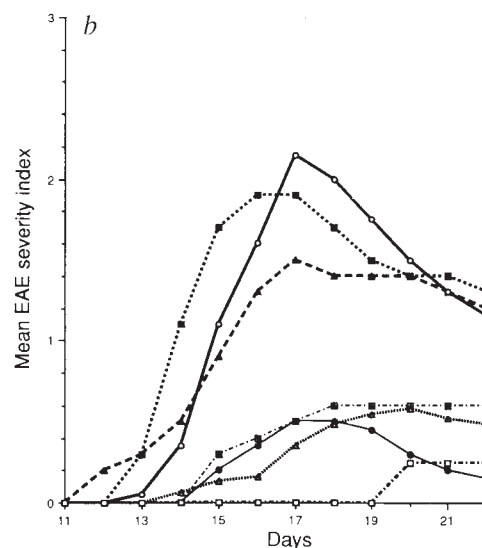
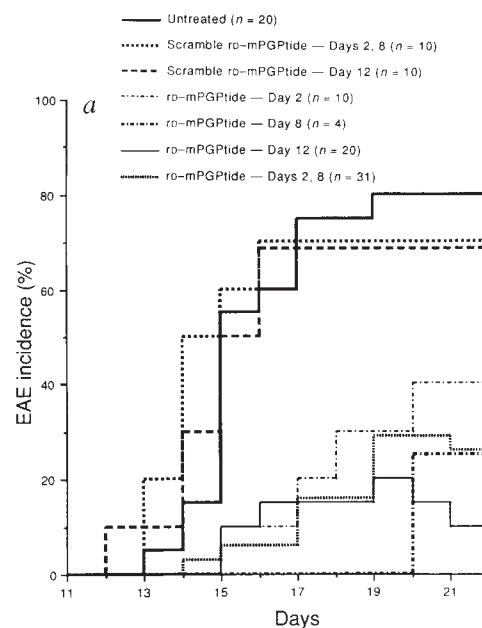


FIG. 2 Effect of CD4 CDR3 region peptide analogues on a mixed lymphocyte reaction (MLR) using ^3H -thymidine incorporation to measure proliferative responses. MLRs were generated as described previously¹⁰. Peptides m86-104 C-C¹¹, mPGPtide¹² and rd-mPGPtide and control peptides were added at the same time responding cells were added to stimulators. Control peptides m86-104 scramble and scrambled rd-mPGPtide have identical amino-acid composition as do the m86-104 C-C and mPGPtide peptides, but have the sequence order scrambled. hPGPtide is a CDR3 analogue derived from the human CD4 protein. It inhibits CD4-dependent human MLRs (data not shown), but is inactive in the mouse system. As described previously²², peptides were synthesized using standard Fmoc chemistry, refolded to enrich for intramolecular disulphide bonding, and purified by HPLC before use in bioassays. The sequences of the synthesized peptides are as follows: m86-104 C-C (CELENRKKEEVLWVKVTC), rd-mPGPtide (CPGPEEK-RNELEC, all D-amino acids), mPGPtide (CELENRKKEPGPC), m86-104 scramble (CWKFTLEVVEKERNEELC), scrambled rd-mPGPtide (CEPKNEL-PERGEC, all D-amino acids), and hPGPtide (CEVEDQKEPGPC).

treated with a single dose of the rd-mPGPtide on either days 2, 8, 12, or on combined days 2 and 8, showed a marked reduction of level of incidence, with a maximum incidence of between 20 and 40%. The mean initial onset time of the few mice that developed symptoms of disease was also later in those treated with



groups ($P > 0.05$). Severity of clinical neurological symptoms in the mice were scored based on a 0–5 grading scale²³. The mean severity grade was calculated using all experimental mice in the group, including those with no clinical symptoms.

FIG. 3 Effect of CD4 CDR3 rd-mPGPtide on the development of EAE incidence (a), severity (b), and severity of individual mice on day 17 after EAE induction (c). Mouse spinal cord homogenate (MSCH) was prepared from a pool of SJL mice, as described previously²³. For induction of EAE, 1 mg MSCH was administered s.c. at two sites on the posterior flank of SJL mice in conjunction with complete Freund's adjuvant (CFA) in a 1:1 ratio with a final volume of 0.15 ml per site. A secondary inoculation, similar to the primary, was administered at 7 days. In data pooled from five separate experiments, the inoculated mice were separated into seven treatment groups involving the i.v. injection of peptides (0.5 mg) in 0.25 ml on the appropriate days. These groups included mice left untreated or injected with either scrambled rd-peptide or rd-mPGPtide on days 2, 8, combined 2 and 8, and 12. For severity, the differences were statistically significant, by Tukey multiple analysis of variance, between the untreated and the rd-mPGPtide day 2,8-treated group on days 15–20 ($0.001 < P < 0.04$); the untreated and the rd-mPGPtide day 12-treated group on days 16–22 ($0.002 < P < 0.015$); the untreated and the rd-mPGPtide day 2-treated group on days 17 ($P = 0.025$); and the untreated and the rd-mPGPtide day-8 treated group on days 17 ($P < 0.05$). There were no significant differences between the two scrambled rd-mPGPtide treatments and the untreated group ($P > 0.05$) or between the individual rd-mPGPtide

the rD-mPGPtide at any of the time points in comparison to the untreated and scrambled peptide-treated groups.

In regard to the clinical expression of disease (Fig. 3b), the mean EAE severity grade of mice reached a maximum score on day 17 of 2.1 ± 0.4 (s.e.m.) for the untreated group, 1.9 ± 0.5 for the scrambled rD-PGtPide group treated on days 2 and 8, and 1.5 ± 0.4 for the same group treated on day 12. In stark contrast, the maximum severity levels reached in either of the groups given rD-mPGPtide was only 0.6 ± 0.2 . A scatter plot of the severity index attained for individual mice on day 17 is shown in Fig. 3c. Histopathological analysis indicated that clinical improvements in the rD-mPGPtide-treated animals correlated with an absence of inflammatory cells in the central nervous system (data not shown).

To investigate whether the rD-mPGPtide could have an effect on the development of EAE once symptoms had already appeared (corresponding to the late effector phase of the disease), 30 SJL mice were inoculated for EAE. On day 16, 12 mice that had reached a grade 1 level of clinical EAE expression were selected and randomly grouped to be left untreated or treated intravenously (i.v.) with 0.5 mg rD-mPGPtide. The untreated group of mice progressed to an average severity of 2.3 ± 0.3 by day 18, whereas those mice treated with rD-mPGPtide reached only a grade of 1.2 ± 0.2 on day 19 and improved rapidly thereafter (Fig. 4).

SJL mice that had been inoculated for EAE and were left either untreated or treated with 0.5 mg i.v. rD-mPGPtide on day 12, were sampled on day 27 for the presence of CD4⁺ and CD8⁺ T-cell subsets in the spleen and lymph nodes. Phenotypes of the cells were determined by flow cytometric analysis with fluorescein isothiocyanate (FITC)-labelled anti-murine CD4 and CD8 antibody. The cellular composition of the lymphoid organs in the two groups was equivalent. There was no significant diminution of CD4⁺ cells in those mice treated with the rD-mPGPtide in comparison to untreated mice (38 versus 42% in lymph nodes; 12 versus 13% in spleen, $P > 0.76$ for both groups). In addition, lymph node cells from the rD-mPGPtide treated group were assayed on day 27 for immune responsiveness in a mixed lymphocyte reaction to irradiated (1,500 cGy) major histocompatibility complex (MHC)-allogeneic stimulator spleen cells (H-2^b haplotype) and gave proliferative responses equivalent to both the untreated and normal control mice (data not shown). These data demonstrate that, in contrast to antibody treatment, the peptide-based therapy causes neither the depletion of the CD4⁺ subset nor the resultant long-term immunosuppression seen in mice treated with anti-CD4 antibody¹⁷.

The behaviour of the analogues in biological assays has suggested a surprising mechanism. The CDR3 peptides do not inhibit the CD4-MHC class II interaction, which is considered to be the central role of CD4. Instead, pretreatment experiments with different cell types clearly indicate that the CDR3 analogues act at the level of the T cell^{11,12}. These data support a mechanism

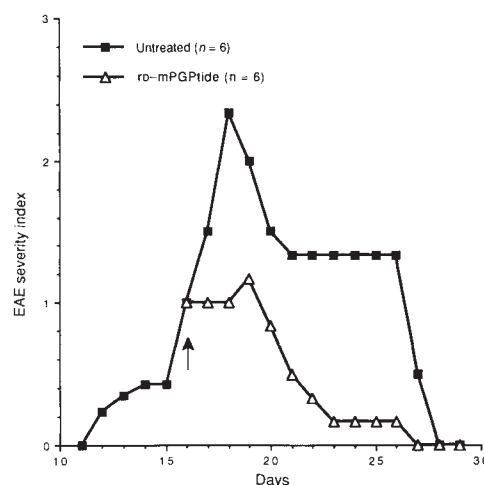


FIG. 4 Effect of rD-mPGPtide on clinical severity of EAE during the late effector phase. Mice were injected on day 16 after EAE induction. At day 18, the difference between the untreated and treated groups was statistically significant by two-way ANOVA ($P = 0.003$).

in which CDR3 analogues act by uncoupling CD4 from the signal transduction machinery, thereby preventing helper T-cell activation.

The use of peptide mimetics of protein surfaces represents an intermediate step between traditional small organic drugs and whole protein therapies. The small size and unnatural chirality of the rD-mPGPtide analogue makes it very poorly immunogenic (we have been unable to detect antibodies directed against it in the sera of peptide-treated mice). Note that the general applicability of our design techniques have limitations. Specifically, the presence of bulky β -substituted amino acids, such as isoleucine and threonine, would have made it difficult to achieve an equivalent surface by simply reversing the direction of the main chain. Furthermore, if the receptor recognition of the analogue had required combinations of side-chain and α -backbone interactions, then our simple approach would probably have been compromised.

Our observed inhibition of the murine EAE disease with peptide analogues of CD4 suggests that a similar approach may be very effective in the treatment of human MS and other CD4-dependent autoimmune disorders^{18,19}. The re-creation of surfaces involved in molecular recognition events offers a powerful new approach in characterizing the molecular interactions of the immune response and may enable the development of new rationally designed immunomodulatory agents. □

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Hox11 controls the genesis of the spleen

Charles W. M. Roberts, John R. Shutter & Stanley J. Korsmeyer

Howard Hughes Medical Institute and Departments of Medicine and Pathology, Washington University School of Medicine, St Louis, Missouri 63110, USA

MANY homeobox genes are clustered in a linear array along a chromosome, reflecting their ordered expression along the anterior-posterior axis of the embryo¹. Expression patterns^{2,3} as well as grafting⁴, ectopic expression^{5,6} and loss-of-function experiments⁷⁻¹¹ suggest that the Hox genes encode a combinatorial system of positional specification along that axis. In contrast, the function of orphan homeobox genes¹² located at sites outside the four mammalian Hox clusters is less well understood. To assess the functional role of the orphan homeobox gene *Hox11*, we have generated *Hox11*-deficient mice through gene targeting. *Hox11*^{-/-} mice have no spleen, but otherwise appear normal. *Hox11* is normally expressed in the splenic anlage arising from the splanchnic mesoderm. *Hox11*^{-/-} embryos have no cellular organization at the site of splenic development but all other splanchnic derivatives develop normally. *Hox11* controls the genesis of a single organ, providing new insight into the genetic regulation of morphogenesis.

HOX11 was originally isolated from the recurrent t(10;14)(q24;q11) chromosomal breakpoint found in human T-cell acute lymphoblastic leukaemia (ALL)^{13,16}. This translocation juxtaposes *HOX11* with a gene encoding the T-cell receptor and redirects the expression of the normal *HOX11* product to thymocytes. Transgenic mice in which *HOX11* is redirected to the thymus develop T-cell lymphoblastic lymphoma-leukaemia, establishing the primary oncogenic importance of this event¹⁷.

Isolation of a murine *Hox11* genomic clone revealed that the homeodomain shared complete amino-acid identity with human *HOX11* and mapped to the syntenic region within the mouse. A homologous recombination replacement vector was created and D3 embryonic stem (ES) cells were electroporated (Fig. 1a). Seven of 163 G418-gancyclovir double-resistant clones had undergone homologous recombination. Clones were injected into blastocysts and reimplanted to generate chimaeric mice. High-colour coat chimaeras transmitted the disrupted allele through the germ line.

Heterozygous mice were completely normal and matings resulted in the expected mendelian frequency of *Hox11*^{-/-} mice which grew into adulthood and were externally indistinguishable from wild-type littermates (Fig. 1b). Both males and females were fertile. We searched for defects in the *Hox11*^{-/-} mice by examining tissues that express *Hox11*. RNA *in situ* hybridization has revealed that *Hox11* is expressed within portions of the developing branchial arches and hindbrain (ref. 18 and C.W.M.R. *et al.*, manuscript in preparation). *Hox11* is initially expressed at embryonic day 8.5 (E8.5) in the developing muscle plates of branchial arches 1, 2 and 3 and subsequently in the motor nuclei that innervate them, cranial nerves V, VII, and IX respectively. Additionally, *Hox11* is expressed in the vestibulo-cochlear, geniculate, and a portion of the trigeminal ganglia, the surface ectoderm of the first branchial arch, and in the ventral portion of the third branchial cleft. Beginning at E11.5, we found that *Hox11* is also expressed at a single site in the abdomen within a portion of the splanchnic mesoderm (Fig. 2a).

Hox11^{-/-} embryos showed no sites of expression by *in situ* hybridization, indicating that the *Hox11* transcript was indeed disrupted (results not shown). Extensive dissection revealed that the pharyngeal and mastication muscles derived from the branchial arches were present and anatomically normal. Moreover, cranial ganglia that express *Hox11* had normal morphol-

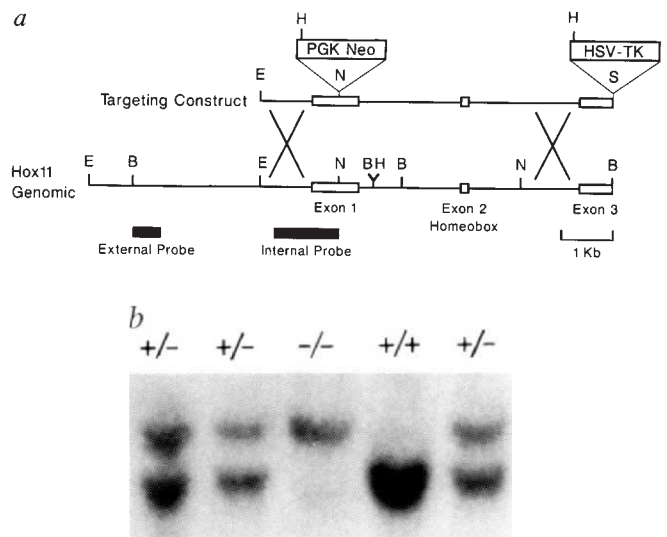


FIG. 1 Homologous recombination of *Hox11*. *a*, Schematic representation of targeting construct and genomic DNA. PGK neo was inserted at a *NarI* site at codon 53 of the open reading frame contained in exon 1. This is upstream of the homeodomain beginning at codon 200 in exon 2. A herpes simplex virus thymidine kinase (HSV-TK) cassette was inserted at the end of the long arm of homology. The external probe detected a 5.2-kb *Bam*HI fragment in germ-line SV129 genomic DNA and a predicted 6.8-kb *Bam*HI fragment after homologous recombination. The external probe hybridized to a 6.3-kb *Hind*III germ-line fragment and a 5.6-kb rearranged fragment after homologous recombination as a result of a *Hind*III site within PGK neo. The internal probe was used to confirm homologous recombinants and ensured that there was only one integration site. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nar*I; S, *Sal*I. The *Sal*I site in the targeting construct is from the poly-linker and is not found in genomic DNA. *b*, Genomic Southern blot showing a *Bam*HI digest of DNA from a *Hox11*^{+/-} × *Hox11*^{+/-} litter hybridized with the external probe.

METHODS. 3×10^7 D3 strain ES cells were electroporated with 30 μ g targeting construct in a volume of 0.8 ml HEPES buffered normal saline at 275 V, 200 μ F in a BTX 300 apparatus (BTX, San Diego, CA) with a flat-pack electrode. 3×10^6 cells were then plated on irradiated neomycin-resistant primary murine embryonic fibroblasts (MEF). At 24 h, 500 μ g ml⁻¹ G418 and 2 μ M gancyclovir were added for selection. Colonies were picked from day 9 onwards, dispersed and transferred to 24-well plates containing irradiated MEF. When colonies appeared, they were picked, dispersed into a single-cell suspension and 75% of the cells were frozen. The remaining 25% were expanded for DNA in 24-well plates on gelatin alone. Homologous recombinants were confirmed with both *Bam*HI and *Hind*III digestion and hybridization with external and internal probes. ES clones with a disrupted *Hox11* allele were microinjected into C57BL/6 blastocysts and reimplanted into 2.5-d pseudo-pregnant females to generate chimaeric offspring.

ogy and position and the cranial motor nuclei appeared to be functioning normally. But complete blood counts revealed that although *Hox11*^{-/-} mice had normal numbers of red blood cells, they had increased numbers of white blood cells, which included both neutrophils and lymphocytes (white blood cells, $16.5 \pm 5.1 \times 10^3$ in *Hox11*^{-/-} versus $8.6 \pm 2.5 \times 10^3$ mm⁻³ in normals; $P < 5 \times 10^{-4}$). Flow cytometry indicated that the B-cell and T-cell profile was normal in thymus, lymph node and peripheral blood. *Hox11*^{-/-} mice had many erythrocytes containing nuclear fragments known as Howell-Jolly bodies (Fig. 3a). These abnormal cells are found most frequently in asplenia¹⁹. Abdominal dissection of each of 34 *Hox11*-deficient mice revealed the complete absence of a spleen (Fig. 3b, c). All other internal organs in the *Hox11*^{-/-} mice were normal in appearance and position.

As ablation of *Hox11* resulted in asplenia, we investigated *Hox11* expression in the abdomen of normal embryos from

E8.5–E16.5. *Hox11* was expressed in a highly localized portion of the splanchnic mesoderm beginning at E11.5 (Fig. 2a). During the eleventh day of embryonic development, cells that express *Hox11* appeared to be emigrating from splanchnic mesoderm and starting to condense in a small mass on the dorsolateral margin of the future stomach (Fig. 2b). This mesoderm continues to express *Hox11* as it proliferates to form the definitive spleen. By E12.5, the splenic primordium has increased in size and is visible as a ridge of cells expressing *Hox11* on the dorsal aspect of the stomach (Fig. 2c). Sections through this area revealed that the spleen had partially separated from the stom-

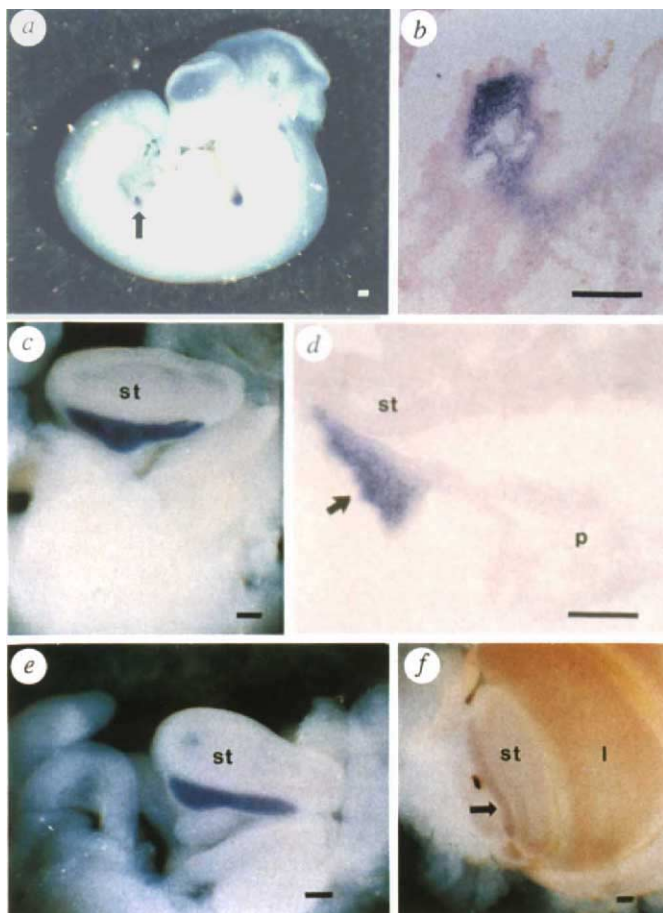


FIG. 2 Expression of *Hox11* in the developing spleen. a, E11.5 whole-mount embryo hybridized with *Hox11* antisense probe. Expression within the splanchnic mesoderm is indicated by an arrow. Also visible is the expression within the branchial motor nuclei in the hindbrain and at the third branchial cleft. b, Transverse section through the splanchnic mesoderm of the E11.5 embryo hybridized with the *Hox11* antisense probe. Mesenchymal cells expressing *Hox11* are beginning to condense at the site of the splenic primordium. c, Abdominal organs from an E12.5 embryo hybridized with a *Hox11* antisense probe. The splenic primordium attached to the dorsal side of the stomach is strongly positive. d, Transverse section through the same E12.5 embryo showing *Hox11* expression in the mesenchymal cells of the splenic primordium. No expression is noted within the stomach or pancreas. e, Abdominal organs from an E13.5 embryo hybridized with a *Hox11* antisense probe. The splenic primordium is still expressing *Hox11*. f, Abdominal organs from an E14.5 embryo hybridized with *Hox11* antisense probe. Expression of *Hox11* in the spleen (arrow) is markedly weaker than at previous times. st, Stomach; p, pancreas; l, liver.

METHODS. Staging of embryos was according to the criteria of Theiler²⁰. Whole-mount *in situ* hybridization was carried out as described³⁰ using a 450-bp probe beginning 3' of the homeobox and continuing into the 3' untranslated region defined by the upstream sequence 5'-AGAGG-AACGTGAGGCCGAGA-3' and downstream sequence 5'-GGATCCAG-AAGCCTCCGG-3'. Abdominal organs of embryos older than E11.5 were cut free before hybridization to maximize probe penetration.

ach and that *Hox11* was specifically expressed in the mesenchymal cells of the spleen (Fig. 2d). *Hox11* was expressed in the developing spleen through E13.5 (Fig. 2e) but was down-regulated thereafter (Fig. 2f). The first haematopoietic cells to populate the spleen do not appear until E15.5 (ref. 20). The first recognized histological evidence of splenic development was the condensation and proliferation of mesenchymal tissues recorded at E12.5 (ref. 21). Consequently, the expression of *Hox11* at E11.5 identifies mesodermal cells destined to form the spleen before the appearance of a morphologically recognizable organ.

The mesogastrium, a precursor of the adult mesentery, is derived from mesoderm and gives rise to the pancreas and spleen. Sections through an E13.5 wild-type mouse revealed an obvious splenic primordium within the dorsal mesogastrium (Fig. 4a). However, serial sections through an E13.5 *Hox11*^{-/-} embryo showed no evidence for a splenic primordium (Fig. 4b). Development of the pancreas and stomach was normal in *Hox11*^{-/-} mice. More caudal sections through earlier E12.5 wild-type embryos identified cellular organization and mesenchymal cell condensation at the site where the spleen forms (Fig. 4c). In contrast, serial sections through E12.5 *Hox11*^{-/-} mice revealed no identifiable organization or condensation and indicated that the effect of *Hox11* disruption was already manifest by E12.5 (Fig. 4d). There was no evidence at earlier time points in *Hox11*-deficient embryos of spleen development. Thus elimination of *Hox11* results in a specific, localized defect in the mesodermal cells normally destined to form the spleen, while other structures within the mesogastrium develop normally.

Our results indicate that a single homeobox gene is specifically required for the genesis of one organ. Genes involved in the ontogenic process from polarity generation in the fertilized egg²² to embryonic axis formation²³ to gastrulation²⁴ and spatial pattern formation²⁵ have all been identified. A combinatorial gene program appears to pattern the evolutionarily ancient and critical elements of the central nervous system and its axial support structures. However, the main genetic events that control formation and patterning of individual organs remain unknown. Each gene participating in organogenesis could play a role in the development of many tissues. It is of interest that *WT-1*-deficient mice fail to develop kidneys but have other abnormalities as well²⁶. Alternatively, following regional specification by a gene network, individual genes might be activated which then direct development of a single organ. The *Hox11*-deficient mice fit the latter model. An orphan homeobox would not be encumbered by other *cis*-acting regulatory elements that were within a tandemly linked cluster. This might relate to patterning a unique, unilateral organ like the spleen which is not mandatory for survival.

Hox11 is also expressed within the hindbrain and branchial arches. No abnormalities were noted in these structures, although subtle defects might not have been detected. It is possible that *Hox11* expression in the branchial region reflects its homeobox evolutionary origin but has no residual function there. All of the homeobox genes targeted so far have had domains of expression that are apparently unaffected in null embryos. Functional redundancy may exist within the branchial arches and hindbrain where *Hox11* loss could be compensated by other genes.

In humans with asplenia, roughly ten per cent of cases have no other associated defects²⁷. These cases are frequently familial and represent candidates for *Hox11* defects. The mouse strain dominant hemimelia also has asplenia²⁸ but has many other defects. *Hox11*-deficient mice provide the best model to pursue the role of the spleen in haematopoiesis and immunity. Moreover, they provide a model system to identify the downstream target genes critical for the genesis of this organ.

Hox11 is one of a series of genes that have been isolated from chromosomal breakpoints in acute lymphoblastic leukaemia (ALL). These genes are either misdirected to lymphocytes or altered as fusion proteins²⁹. They include another homeobox gene, *PBX1*. *MLL* (*HRX*, *ALL1*) is a homologue of the *Drosophila*

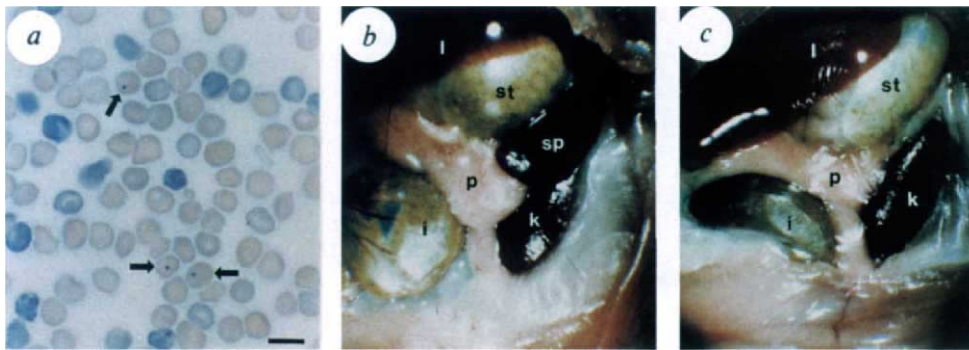


FIG. 3 *Hox11*^{-/-} mice are asplenic. a, Peripheral blood smear from a postnatal day 4 *Hox11*^{-/-} mouse showing three erythrocytes containing Howell-Jolly bodies in one high-powered field (arrows). b and c, Abdominal dissections of normal (b) and *Hox11*^{-/-} mice (c). The spleen is absent in the *Hox11*^{-/-} mouse. i, Intestine; k, kidney; l, liver; p, pancreas; sp, spleen; st, stomach.

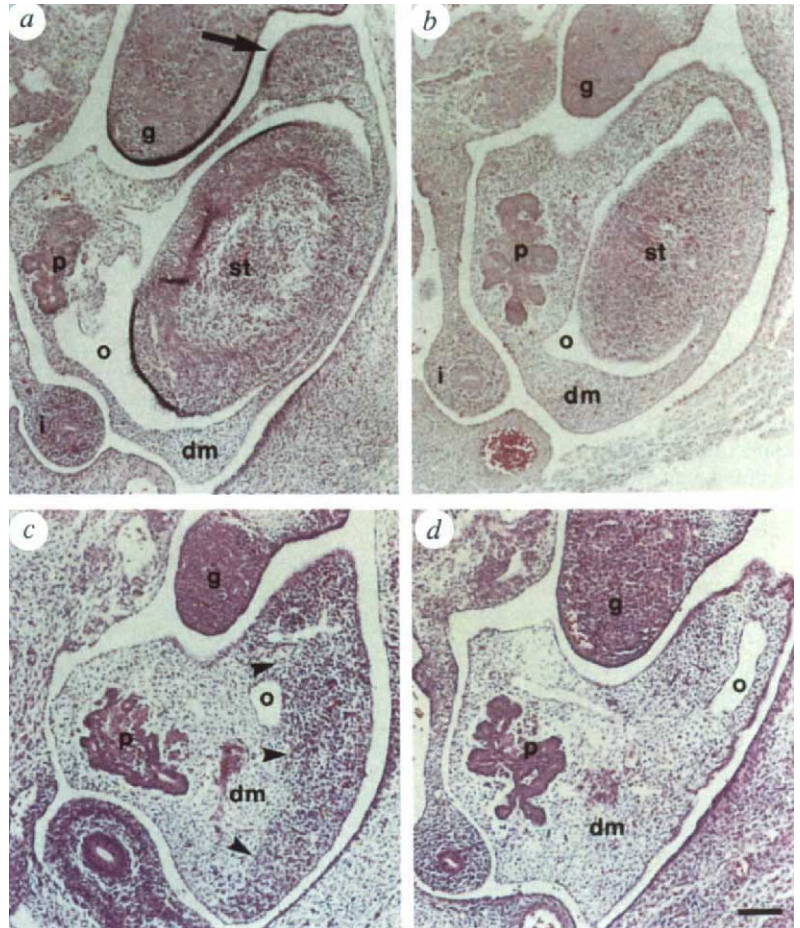


FIG. 4 Histology of normal and *Hox11*^{-/-} embryos. a, Transverse section through an E13.5 normal embryo demonstrating the splenic (arrow) and pancreatic primordia within the dorsal mesogastrium. b, Transverse section through an E13.5 *Hox11*^{-/-} embryo showing the lack of a splenic primordium. c, Transverse section of an E12.5 normal embryo through the dorsal mesogastrium just caudal to the stomach. Arrowheads indicate the boundary of condensation of mesenchymal cells destined to form the spleen. d, Transverse section through an E12.5 *Hox11*^{-/-} embryo demonstrating a lack of condensation and organization of mesenchymal cells within the dorsal mesogastrium. Note the lack of tissue between the sac of the omentum (o) and the lateral edge of the dorsal mesogastrium compared to the normal embryo. dm, Dorsal mesogastrium; g, gonad; i, intestine; o, sac of omentum; p, pancreas; st, stomach.

phila gene Trithorax, which is responsible for homeotic gene regulation. *TTG1* and *TTG2* (*RBTN1*, *RBTN2*) possess LIM domains and related genes are required in lineage development. These transcription factors may prove to be master regulatory genes controlling morphogenesis. During embryonic development, patterning events require extensive cell growth. Redirecting such genes to developing leukocytes could result in rapid proliferation and subsequent leukaemia. □

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Taxol inhibits progression of congenital polycystic kidney disease

David D. L. Woo*, Steven Y. P. Miao*,
Juan C. Pelayo† & Adrian S. Woolf‡

Departments of * Medicine and † Paediatrics, 7-155 Factor Building, UCLA School of Medicine,

Los Angeles, California 90024-1689, USA

‡ Developmental Biology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

POLYCYSTIC kidney diseases (PKD) are the most common hereditary diseases of the human kidney and account for ten per cent of patients requiring renal transplantation or dialysis. Renal cyst formation has been attributed to enhanced cell proliferation, unbalanced cell death, abnormal targeting of membrane proteins, aberrant kidney development and tubular obstruction, but there is no treatment that blocks the formation and enlargement of renal cysts. We have now developed an *in vitro* model of spontaneous cyst formation that distinguishes polycystic kidney epithelium from its normal counterpart. Inhibitors of DNA, RNA and protein synthesis did not prevent *in vitro* cyst formation, but this was reversibly inhibited by ouabain, amiloride and the microtubule-specific agents colchicine, vinblastine and taxol. The *cpk* mouse is a well-characterized recessive PKD model¹⁻³ and we find that *cpk/cpk* mice develop PKD and die from uraemia by 4-5 weeks of age, but when treated weekly with taxol they survive for more than 200 days with minimal loss of renal function, show limited collecting-duct cyst enlargement, and attain adult size. Our results indicate that the microtubule cytoskeleton has a central role in the pathogenesis of PKD in *cpk* mice and that taxol may also be useful in treating human PKD.

Normal and polycystic human kidney cells can be cultured as monolayers *in vitro*⁴⁻⁷, and when embedded in type-I collagen, both cell types can be induced to form cysts with serum or forskolin⁸ or epidermal growth factor⁹. We report here that, when placed in a stationary suspension culture system free from the influences of glomerular filtration, renal tubular obstruction and uraemia, epithelial cells from human and murine polycystic kidneys spontaneously develop into cysts (Fig. 1) in a process that superficially resembles the formation of the blastocoele cavity¹⁰. The ability of primary cells from polycystic kidneys to form cysts *in vitro* indicates that they possess intrinsic morphogenetic information that is absent in normal kidney cells.

Methotrexate (10 µg ml⁻¹), cytosine arabinoside (20 µg ml⁻¹), α -amanitin (100 ng ml⁻¹) or cyclohexamide (40 µg ml⁻¹) did not inhibit cyst formation, suggesting that DNA, messenger RNA and protein synthesis are not required for cyst formation in this *in vitro* model. Sodium and proton transport are the principal forces responsible for the transepithelial movement of water in kidney tubules and we found that amiloride (1 mM) or ouabain (1 mM) reversibly blocked cyst formation *in vitro*, suggesting that fluid accumulation is integral to cyst formation. Taxol (25 µM), colchicine (10 µM) and vinblastine (1 µM) all reversibly inhibit *in vitro* cyst formation, suggesting that microtubules have a functional role in this model.

Although the model distinguishes normal and PKD epithelia, the topology of cysts formed in suspension have their basolateral surfaces facing the lumen. This topology is the opposite of *in vivo* renal cysts which have luminal apical surfaces. We therefore tested the validity of the model by studying the effect of microtubule-specific inhibitors on the *in vivo* development of renal cysts in a well-characterized recessively inherited model of PKD, the *cpk* mouse¹⁻³. First, polycystic *cpk* mice were given a single interperitoneal taxol treatment between the tenth and the twelfth postnatal day. This age is the earliest time when cystic kidneys

can be palpated and it coincides with the onset of cyst development within collecting ducts¹⁻³. Untreated polycystic mice ($n=6$) or polycystic mice treated with dimethylsulphoxide (DMSO) alone ($n=6$) died between 25 and 28 postnatal days. However, polycystic mice treated once with 150 µg taxol between days 10-12 ($n=11$) all survived to 40-50 days old, an increase of 6-9 standard deviations. Minimum doses of colchicine (250 nM), colcemid (200 nM) and vinblastine (200 nM) required to disassemble microtubules *in vitro* are lethal to 10-12-day-old polycystic *cpk* mice (intraperitoneal LD₅₀s of colchicine, colcemid and vinblastine in adult mice are 2 mg kg⁻¹ (5 µM), 35 mg kg⁻¹ (87 µM) and 1 mg kg⁻¹ (1.1 µM), respectively), whereas sublethal doses of these compounds are ineffective in prolonging the survival of *cpk* mice.

As taxol turns over *in vivo*, we next tested the effect of weekly taxol treatment on the development of PKD in *cpk* mice. The survival of 11 polycystic *cpk* mice given weekly taxol treatments in one experiment is shown in Fig. 2. Histological examination of taxol-treated polycystic kidneys at up to 200 days revealed the presence of normal glomeruli and normal to slightly dilated renal tubules in both the kidney cortex and medulla (Fig. 3i-p). Although cysts were present in all the taxol-treated polycystic kidneys, they were less numerous and less dilated than cysts found in 21-day-old untreated polycystic kidneys. By contrast, normal tubules and glomeruli are absent in end-stage *cpk* kidneys (Fig. 3g, h, s), suggesting that the collecting duct cyst formation and expansion phase of the disease had been arrested or greatly retarded. Fibrosis of the kidney interstitium, a feature commonly found in human polycystic kidneys, was evident in taxol-treated polycystic mice (Fig. 3r), but fibrosis was not detected in untreated *cpk* kidneys; we speculate that the rapid onset of uraemic death precludes this feature. Chronic fibrosis of the renal interstitium may lead to worsening of renal insufficiency and to eventual renal failure. It is well known that if a kidney is damaged by any one of a wide range of factors (immunological or ischaemic, for example), and this insult is subse-

TABLE 1 Progression of polycystic kidney disease in *cpk* mice with or without weekly taxol treatment

Age (days)	Treatment	Body weight (g)	Kidney weight (g)	Creatinine (mg dl ⁻¹)
10	None	5.3 ± 0.6	0.15 ± 0.02	0.56 ± 0.1
14	None	6.4 ± 0.7	0.47 ± 0.1	0.51 ± 0.1
21	None	7.4 ± 0.7	1.4 ± 0.2	1.58 ± 0.1
28	None	10.4 ± 1.9	1.9 ± 1.3	2.54 ± 0.3
25	Taxol	8.8	0.4	0.88
45	Taxol	11.8	1.2	2.07
62	Taxol	12.8	2.2	1.22
92	Taxol	15.5	1.7	1.16
120	Taxol	17.3*	1.4*	0.93*
182	Taxol	21.7*	0.5*	0.84*
210	Taxol	21.5*	0.6*	0.87*

Average serum creatinine of 200-day-old normal mice is 0.64 ± 0.11 mg dl⁻¹ ($n=6$). All data shown from untreated mice are an average of $n=3$ at each time point; data from treated mice refer to a single animal at that time. The kidney weights of taxol-treated mice are significantly less than the weight of untreated polycystic *cpk* kidneys, suggesting that the gross progression of polycystic kidney disease is retarded or arrested by taxol. When available for assay, the serum creatinine levels from mice that died before 120 days of age are also significantly lower than those found in untreated *cpk* mice with end-stage PKD. This moderate level of uraemia, together with the presence of significant numbers of normal glomeruli and tubules, suggests that these mice probably died of a combination of taxol toxicity and renal insufficiency, and not of kidney failure from PKD alone. Serum proteins were precipitated with 5% trichloroacetic acid and removed by centrifugation. Deproteinized serum was then neutralized to pH 7 with NaOH. Serum creatinine levels were determined using 25 µl neutralized deproteinized serum in a 250-µl reaction using the fading fraction method²⁷.

* Data are an average of $n=2$.

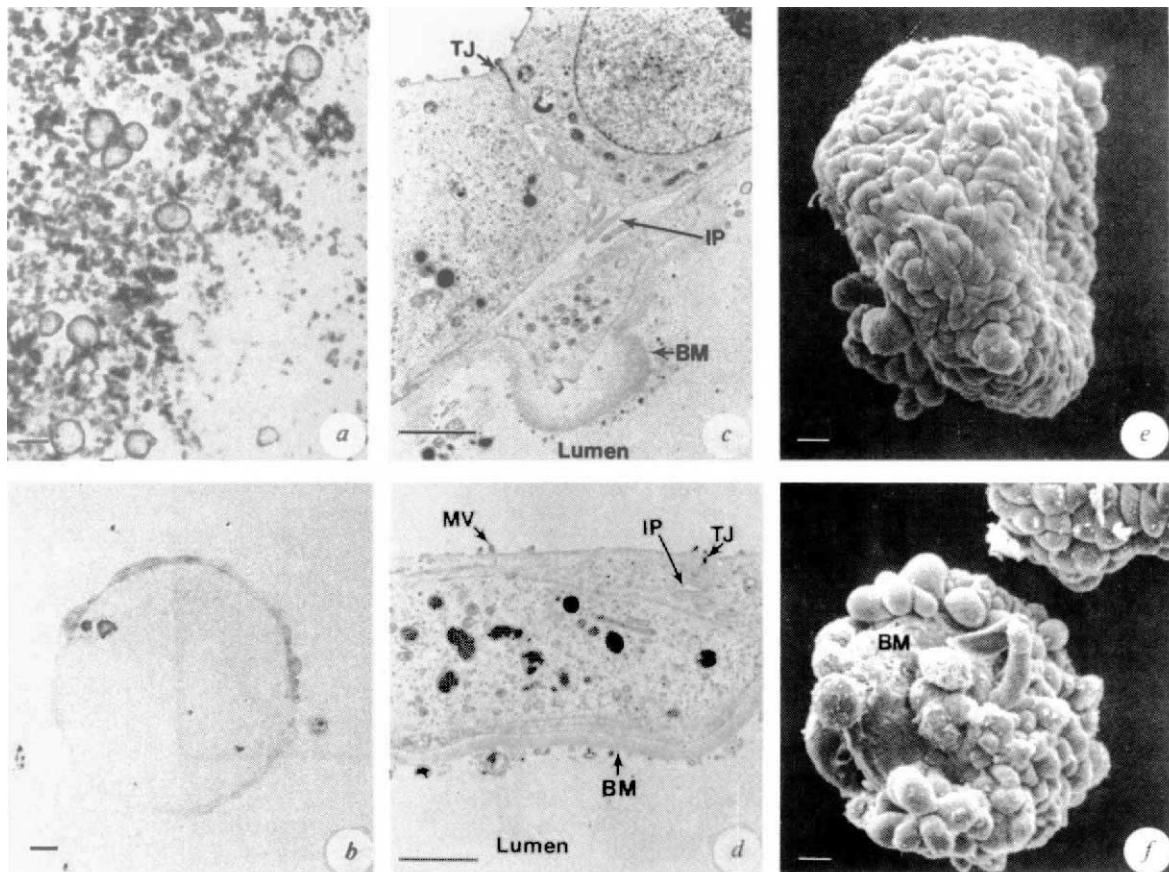
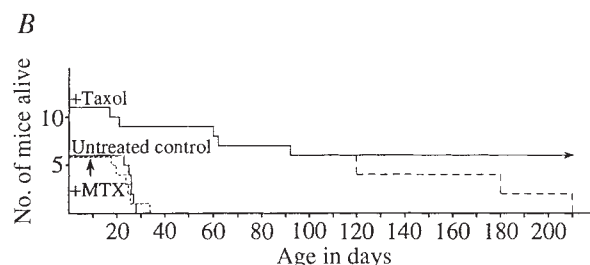
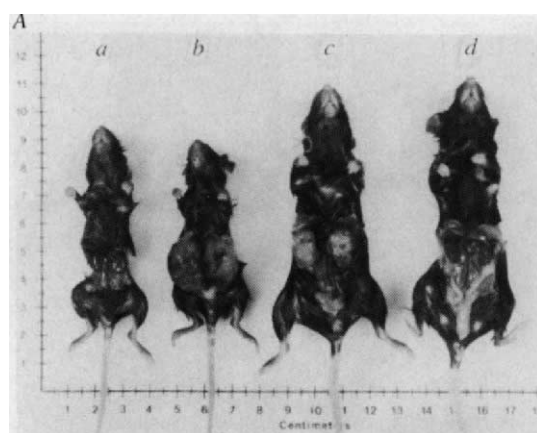


FIG. 1 *In vitro* formation of cysts from polycystic kidney cells. *a-f*, Human samples. In stationary suspension culture, dispersed polycystic human kidney cells aggregate into clusters of 50–100 cells within 4 h. By 24 h, microscopic lumens are visible. The lumens gradually enlarge and reach a steady-state size of 50–200 μm in diameter by day 7, when intraluminal hydrostatic pressures reach 10 mm Hg (*a*). Cysts form in both serum-free and serum-containing medium with similar efficiencies and can be maintained in culture for up to 6 weeks. Normal human kidney cells form aggregates but small lumens are only rarely found. Cysts are one cell thick (*b*), ranging over 10–50 cells in circumference. *e*, Scanning electron micrograph (SEM) of a large 'deflated' cyst. In cysts in which some cells have become detached (*f*), the basement membrane is visible. Cells lining the cysts are polarized, have apical tight junctions and abnormally thickened basement membrane on the basal surface (*c, d*). (TJ, tight junction; IP, interdigitating processes; BM, basement membrane; MV, microvilli.) Scale bars: *a*, 50 μm ; *b, e, f*, 10 μm ; *c, d*, 2 μm . *g-j*, *cpk* mouse samples. *g*, After 24 h, normal mouse kidney cells remained either as single cells, aggregates of a few cells, or aggregated into groups of 20–80 cells. *h*, Both cysts and aggregates have formed in cultures of *cpk* kidney cells by 24 h. Lumens could first be identified after 6 h. Typically, 400–800 cysts developed from 1×10^6 viable cells. *i*, A typical cyst ($\times 130$). *j*, Apical microvilli and tight junction (arrow) are found on the apical surface of cyst cells.

METHODS. Human samples: each gram of kidney tissue from either normal ($n=9$) or polycystic human kidneys ($n=10$) was trimmed into 1–3 cubes and processed as described below. Typically, 100 to 150 cysts can be prepared from 1 g of polycystic kidney. Mouse samples: kidneys were removed at 10, 21 and 28 days after birth from *cpk* mice and their normal littermates. Kidney pieces were incubated at 39 °C for 1–3 h in 10 ml of collagenase type IV (1 mg ml^{-1}) containing egg-

white trypsin inhibitor (1 mg ml^{-1}) in DMEM/F12. Tissue dissociation was aided by vigorous shaking at 15–30-min intervals. The preparation was then centrifuged at 200g for 1 min. Pellets were resuspended in 14 ml DMEM/F12 with or without 10% calf serum. Larger aggregates were removed by settling under gravity for 2 min. The top 12 ml, which contained single cells and aggregates of up to 10 cells, were placed in the wells of plastic culture plates precoated with a 3-mm layer of 1% agarose in DMEM:F12. Plating cell density is 1×10^6 cells in 2 ml per well for a 6-well plate, and 3×10^5 cells in 0.5 ml per well for a 24-well plate. Cell suspensions were incubated at 37 °C in a moisture-saturated incubator in 5% CO_2 and air.

FIG. 2 A, Taxol prolongs the survival of polycystic *cpk* mice. 28-Day-old normal (a) and polycystic (b) *cpk* mice are shown next to polycystic (c) and normal mice (d) treated weekly with taxol for 120 days. Taxol-treated polycystic kidneys are smaller and less cystic. B, Survival of untreated, methotrexate- or taxol-treated polycystic *cpk* mice. Untreated and methotrexate-treated (MTX) polycystic *cpk* mice all died before 30 d of age. The survival of some polycystic *cpk* mice treated with weekly taxol can be prolonged to more than 60 days. The dotted line starting at day 120 indicates the number and age of apparently healthy taxol-treated polycystic mice killed for histology and serum creatinine determinations. Two mice died at 18 and 21 days from taxol toxicity. Despite having palpably enlarged kidneys, the 6 mice remained active until they were killed after day 20 and attained adult size and weight. This experiment was repeated twice with comparable results. Five out of 11 taxol-treated polycystic *cpk* mice survived over 120 days before the apparently healthy animals were killed in one experiment, and 5 out of 12 polycystic mice are still alive and



appear healthy after >150 days in a current experiment. A log-rank χ^2 of 8.14 was calculated for the difference between the control and taxol-treated *cpk* mice ($P=0.0043$). The survival of methotrexate-treated, DMSO-treated and untreated *cpk* mice is statistically insignificant ($P>0.05$).

METHODS. Starting at 10–12 days of age, each polycystic offspring of heterozygous *cpk* mice was given, on a weekly basis, either 15 μ l taxol (10 mg ml⁻¹ in DMSO) or 15 μ l methotrexate (1.1 mg ml⁻¹ in saline), or 15 μ l DMSO alone, intraperitoneally. All juvenile animals were kept with their mother, weighed every other day and weaned when they were 25 days old.

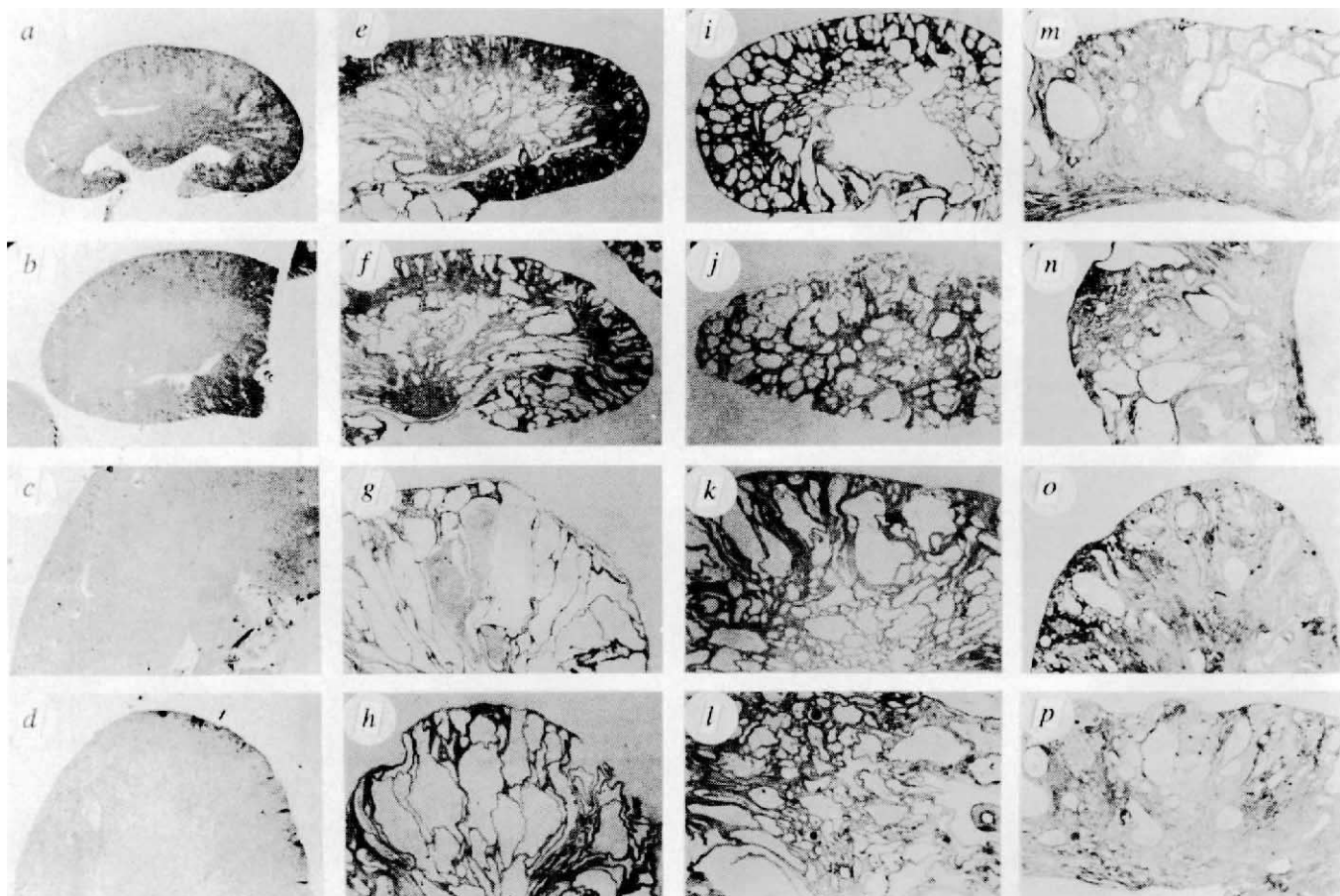
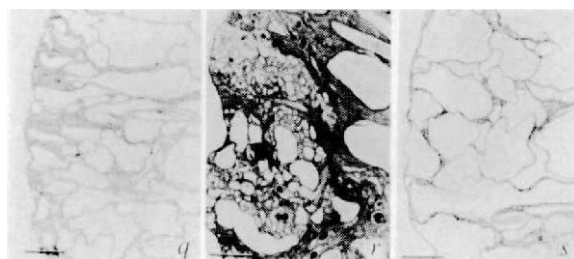


FIG. 3 Cross-section of normal, polycystic and taxol-treated polycystic kidneys from *cpk* mice. a–d, Normal kidneys: a, day 10; b, day 14; c, day 28; d, day 120. e–h, Untreated polycystic kidneys: e, day 10; f, day 14; g, day 21; h, day 28. i–p, Taxol-treated polycystic kidneys: i, day 25; j, day 45; k, day 62; l, day 92; m, day 102; n, day 182; o, p, day 210. $\times 6$. Sections are stained with periodate–Schiff stain counterstained with haematoxylin. The extent of cyst formation in day-14 and day-28 (end-stage) untreated *cpk* kidneys are shown in q and s, respectively. Between days 14 and 28, untreated *cpk* kidneys suffered from rapid expansion of cysts and loss of numerous tubules. Interstitial fibrosis is absent in untreated *cpk* kidneys. In the kidney from a *cpk* mouse treated for 210 days with taxol, shown in r, cystic expansion and loss of renal tubules are either blocked or greatly retarded. Note that the remaining tubules are much larger because of hypertrophy. Generalized interstitial fibrosis has also developed. Scale bar, 0.5 mm.



quently removed, then progressive renal failure, characterized by glomerular sclerosis, interstitial fibrosis and uraemia, can follow¹¹. We suggest that the fibrosis in treated kidneys results from damage sustained before taxol was administered. Histological examination of the brain, heart, lung, liver and spleen of taxol-treated polycystic mice did not reveal any gross abnormalities. Normal control mice ($n=6$) treated for 200 days with weekly doses of taxol have normal kidney histology. In addition to the survival data and renal histology, the retardation of disease progression in taxol-treated *cpk* mice was confirmed by measurements of the kidney weight, body weight and serum creatinine levels (Table 1).

Because taxol interferes specifically with microtubule functions¹², it has antimitotic properties. Inhibitors of DNA synthesis were therefore evaluated for their ability to inhibit *cpk* cyst formation *in vivo*. Weekly interperitoneal injection of 15 μ l of 1.1 mg ml⁻¹ methotrexate produced a reduction in growth comparable to that in mice treated with 15 μ l of 10 mg ml⁻¹ taxol. This sublethal weekly methotrexate treatment had no effect on the survival of polycystic *cpk* mice (Fig. 2). Sublethal doses of cytosine arabinoside (ara-C) were also ineffective (data not shown). Moreover, as doses of methotrexate and ara-C sufficient to inhibit DNA synthesis did not block lumen formation *in vitro*, the increased proliferative potential of PKD epithelia¹³⁻¹⁶ probably does not play an important part in cyst formation but may generate cells to line expanding cysts.

Apart from their role in mitosis, microtubules contribute to and maintain cellular architecture. They also participate in membrane vesicle trafficking¹⁷, exocytosis and endocytosis¹⁸, and the movement of intermediate vesicles between the endoplasmic reticulum and Golgi^{19,20}. During the formation of trophoblast, microtubules play important parts in the biogenesis of epithelial polarity^{21,22}. After polarity is established, specific pathways deliver secreted and membrane proteins to their target membrane domains and, although these sorting processes are not well characterized, microtubules are clearly involved^{23,24}. Our data implicate the microtubule network in the genesis of PKD cysts both *in vitro* and *in vivo*. It remains to be determined whether altered tubulins or altered microtubule-associated proteins are primary lesions in *cpk* mice or other forms of PKD. It is feasible that aberrations of cellular functions mediated by microtubules may lead to the apical mislocation of Na⁺, K⁺-ATPase and epidermal growth factor receptors in both human²⁵ and murine PKD²⁶. Finally, the ability of taxol to prevent the uraemic death in a mouse model for PKD suggests that taxol or its analogues may be useful in the treatment of human polycystic kidney diseases. □

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Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers

Tsutomu Nobori*, Kaoru Miura*, David J. Wu*, Augusto Lois*, Kenji Takabayashi† & Dennis A. Carson*

* Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0663, USA

† CIBA-GEIGY Pharmaceuticals Division, CH-4002 Basel, Switzerland

CYTOGENETIC abnormalities of chromosome 9p21 are characteristic of malignant melanomas^{1,2}, gliomas³, lung cancers⁴ and leukaemias⁵. From a panel of 46 human malignant cell lines, we localized by positional cloning the most frequently deleted region on 9p21. Sequence analysis of the isolated fragment reveals two open reading frames identical to the recently described complementary DNA for the inhibitor of cyclin-dependent kinase 4 (CDK4)⁶. Polymerase chain reaction and Southern blot analysis confirmed the frequent deletion or rearrangement of the CDK4-inhibitor gene in melanomas, gliomas, lung cancers and leukaemias, and the absence of detectable gene transcripts. One carcinoma had a deletion entirely within the CDK4-inhibitor gene. The CDK4-inhibitor gene from a patient with dysplastic nevus syndrome had a germline nonsense mutation. The CDK4 inhibitor is thought to be a physiological suppressor of proliferation. Cells unable to produce the inhibitor may be prone to neoplastic transformation.

Many malignant cell lines with chromosome 9p21 deletions either lack the enzyme methylthioadenosine phosphorylase (MTAP), or have hemizygous or homozygous deletions of the interferon- α (*IFN- α*) gene cluster^{5,7,8}. The T98G glioma cell line has a small 9p deletion centromeric to the *IFN- α* locus, but has normal methylthioadenosine phosphorylase activity³. These results suggested that the putative tumour-suppressor gene on chromosome 9p resided between the *MTAP* and *IFN- α* loci.

MTAP cDNA was isolated and used to probe a human placenta λ -phage library. A 2-kilobase (kb) *HindIII* fragment (clone 7-2) contained the 3' end of the *MTAP* gene by sequence analysis. Chromosome walking was performed, starting with the 3' end of *MTAP*. Several screening cycles of P1 phage⁹, and subsequent λ -phage libraries led to the isolation of clones encompassing the deleted region in T98G. Restriction fragments of these phage were subcloned, partially sequenced, and mapped by Southern blotting and pulsed-field gel electrophoresis. Figure 1 shows the map of human chromosome 9p21 between the *MTAP* and interferon- β (*IFN- β*) gene loci, focusing on the deleted segment in T98G.

The polymerase chain reaction (PCR) was used to determine the frequency of deletion of several-tagged sites (STS) from chromosome 9p in 46 different human malignant cell lines (Table 1). Depending on the cell type, either STS *54F* or STS *5BS* was deleted most frequently. These results focused our attention on the 50-kb region between STS *54F* and STS *5BS*.

Eight malignant cell lines with breakpoints between *54F* and *5BS* were then analysed by STS-PCR, with new probes from the intervening region. The deletion maps are shown at the

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TABLE 1 Homozygous loss of chromosome 9p loci in human cancer cells

			Frequency of negative STS-PCR (%)								
Cell line (number)		<i>MTAP</i>	3.21	2 <i>F</i>	54 <i>F</i>	<i>CDK4I</i>	5 <i>BS</i>	71 <i>F</i>	3.3 <i>B</i>	<i>IFN-α8</i>	<i>IFN-β</i>
Melanoma	(13)	30.8	38.5	53.8	53.8	61.5	61.5	61.5	15.4	7.7	0
Glioma	(8)	62.5	75.0	87.5	87.5	87.5	75.0	75.0	62.5	62.5	25.0
Lung cancer	(11)	27.3	27.3	27.3	27.3	36.4	36.4	36.4	9.1	9.1	0
Leukaemia	(14)	50.0	50.0	64.3	64.3	64.3	57.1	57.1	28.6	28.6	21.4

Homozygous deletion of these loci was detected by STS-PCR. PCR, except for *CDK4I* and *CDK4I3'*, was carried out in a total volume of 20 μ l, containing 0.1 μ g of genomic DNA, 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin), 200 μ M of each dNTP, 20 ng each of sense and antisense primers, and 0.5 units of *Taq* DNA polymerase. Thirty cycles were performed, each cycle consisting of denaturation (94 °C, 1 min), annealing (50 °C or 55 °C, 1 min), and extension (72 °C, 1 min). 10- μ l aliquots were resolved on 2% agarose gels. The locations of the STS-PCR sites are shown in Fig. 1a. *CDK4I*-PCR was performed as described for Fig. 2.

bottom of Fig. 1. A 19-kb λ -phage clone (10B1) identified the most frequently deleted site. We concluded that the 10B1 phage should contain part of the putative tumour-suppressor gene.

The sequence of a plasmid subclone (10B1-10) from 10B1 contained a 306-base-pair (bp) open reading frame with sequence identity to the 3' region of the recently described cDNA for the human cyclin-dependent kinase-4 (CDK4) inhibitor, apart from the last 15 bp⁸ (Fig. 2a). The 3' end of the coding region, and the 3' non-coding region, was located 2.6 kb towards *MTAP*. The 5' end of the gene was telomeric to the deleted region in T98G (Fig. 1). We rescreened the 46 malignant cell lines with STS-PCR primers, corresponding to the isolated frag-

ment of the CDK4-inhibitor gene, designated *CDK4I* in Table 1. Sixty-one per cent of melanomas, 87% of gliomas, 36% of non-small-cell lung cancers, and 64% of leukaemias have homozygous deletions of the *CDK4I* gene fragment. Melanoma cell line WM266-4 has only the 5' end of the CDK4-inhibitor gene deleted. It was positive for STS *CDK4I*, negative for STS *5BS*, and produced an abnormal 7.0-kb band after *Eco*RI digestion, electrophoresis and hybridization to a probe from the 5' region of the CDK4-inhibitor gene (Fig. 2c). On the other hand, melanoma cell line SK-MEL-31 has only the 3' end of the CDK4-inhibitor gene deleted. It was negative for STS *CDK4I* (Fig. 2b), but produced a normal 4.0-kb band, indistinguishable from

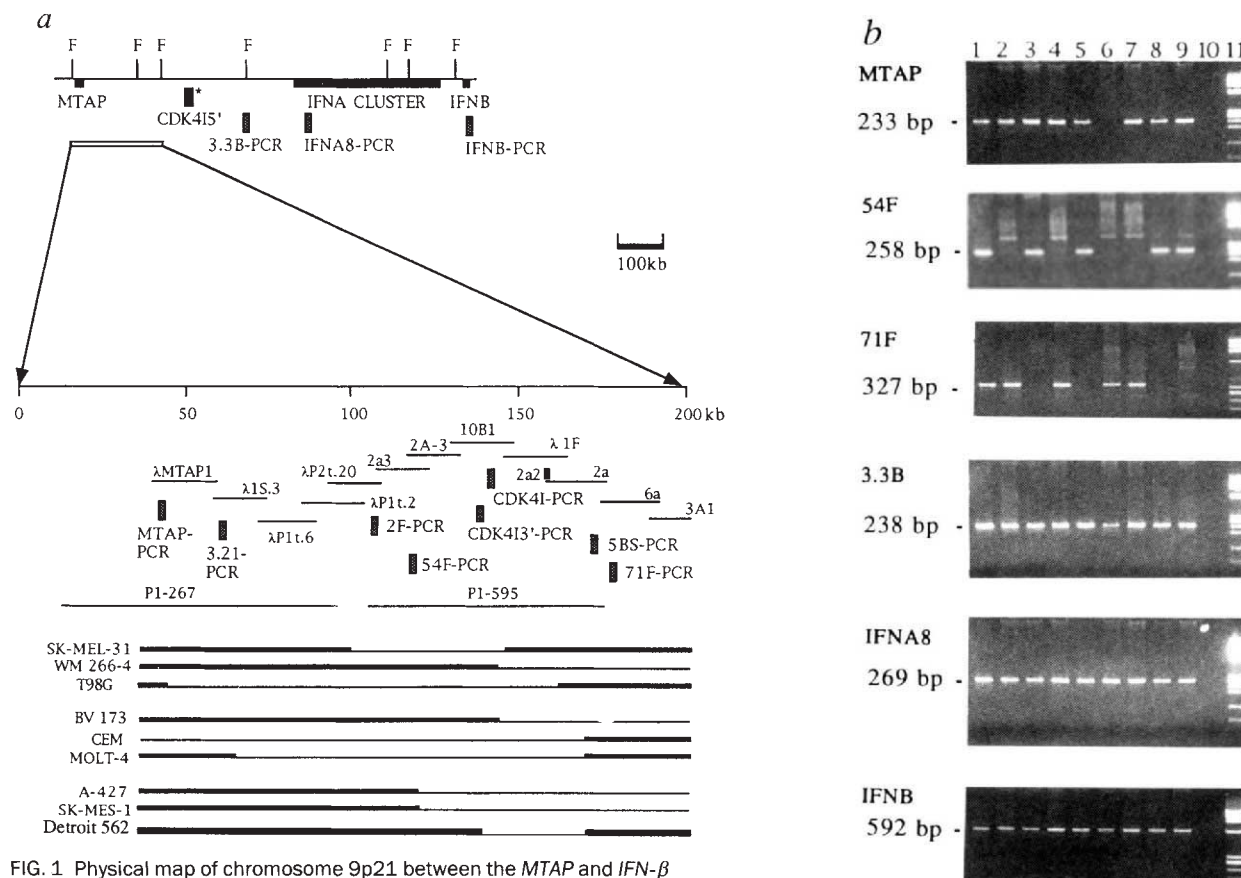


FIG. 1 Physical map of chromosome 9p21 between the *MTAP* and *IFN- β* gene loci. a, The P1 phage clones and λ -phage clones are indicated by thin lines. The sequence-tagged sites (STSs) used for PCR are shown as cross-hatched bars. The *CDK4I* and *CDK4I3'* sequences come from phage 10B1 and are separated by a 2.6-kb intron. These 11 STS-PCR assays localized the approximate breakpoints in the nine malignant cell lines with the most informative deletions. SK-MEL-31 and WM 266-4 are melanomas; T98G is a glioma; BV173, CEM, and MOLT-4 are leukaemias; A-427 and SK-MES-1 are non-small-cell lung cancers; Detroit 562 is a pharyngeal carcinoma. Homozygous deletions are indicated by thin horizontal lines. Asterisk indicates that the location of *CDK4I5'* in the 190-kb *Sfi*I fragment is not precise. F, *Sfi*I. b, STS-PCR analysis. All STS-PCR amplified fragments of the expected size in J640-51 cells containing a single human chromosome 9 on a Chinese hamster background (data not shown). Lanes: 1, human placenta; 2,

SK-MEL-31; 3, WM 266-4; 4, T98G; 5, BV173; 6, CEM; 7, MOLT-4; 8, A-427; 9, SK-MES-1; 10, no template DNA; 11, DNA markers.

METHODS. a, The λ MTAP1 clone was obtained by screening a human placenta λ FIX II genomic library (Stratagene) with *MTAP* cDNA. A 2-kb *Hind*III fragment containing the 3' end of the *MTAP* gene was used for chromosome walking in human P1 phage (Genome Systems), λ -phage (Stratagene), and chromosome-9-specific charon-40 phage (LL09NL01, ATCC) libraries. Clones encompassing the 190-kb region were isolated, subcloned, partially sequenced, and mapped by southern and pulsed-field-gel blotting. b, STS-PCR was developed on the basis of the partial sequences of the subclones. PCR amplification was done under conditions described in Table 1. PCR products were resolved on a 2% agarose gel.

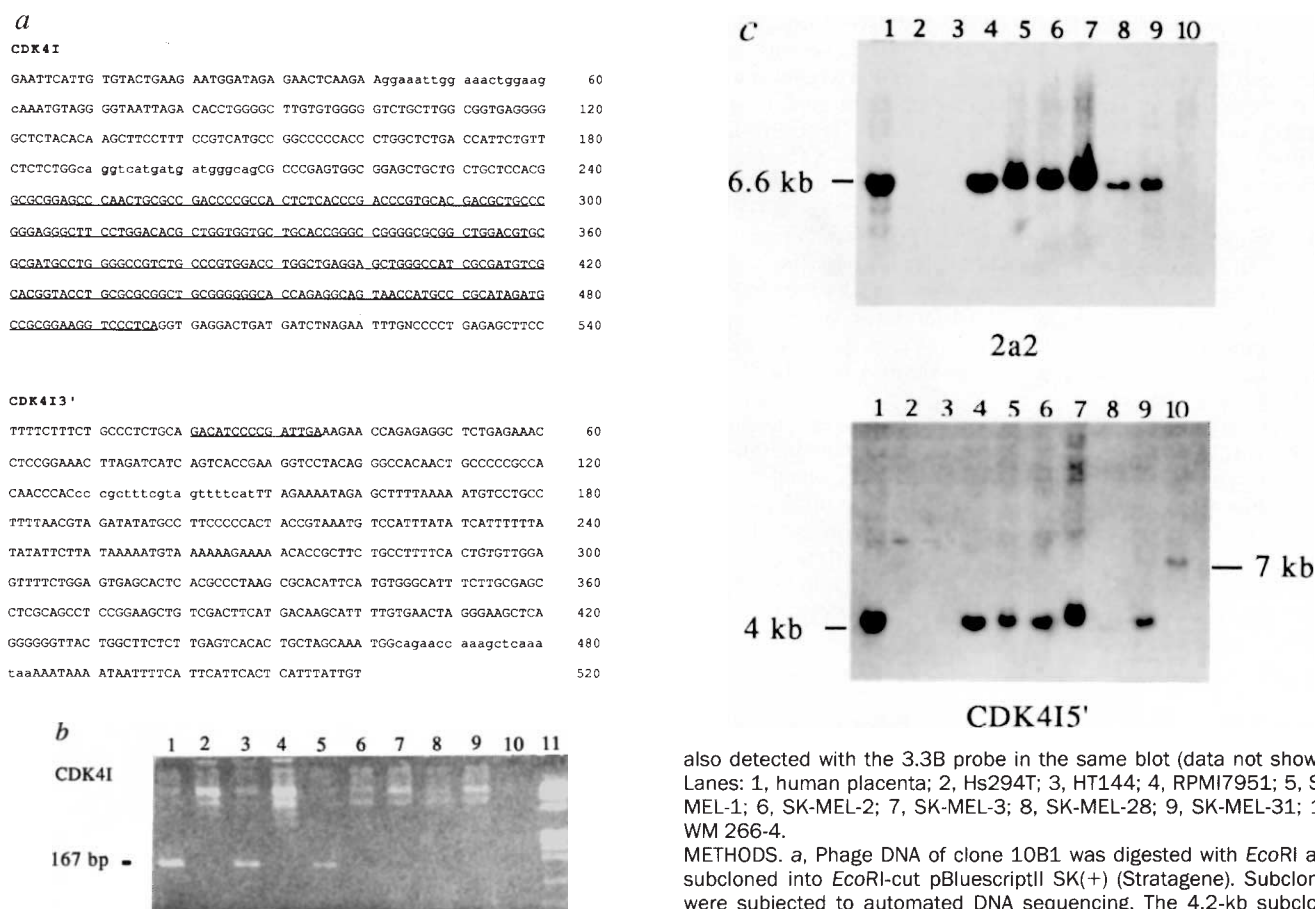


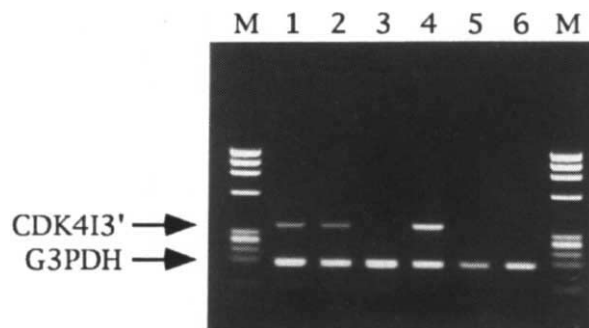
FIG. 2 Absence or rearrangement of the CDK4-inhibitor gene in malignant cell lines. **a**, Partial sequence of the genomic CDK4-inhibitor gene. The genomic sequence designated *CDK4I* has a 306-bp open reading frame with complete sequence identity to CDK4-inhibitor cDNA, from nucleotides 192 to 497 (underlined). The *CDK4I3'* sequence has a short open reading frame (underlined) corresponding to the last 15 bp of the coding region, with a stop codon and the 3' non-coding sequence, except for two nucleotides (T at 197 and A at 493). **b**, PCR analysis of malignant cell lines. Primers for *CDK4I*, shown as lower-case letters in **a**, were used to amplify an expected 167-bp product in human placenta (lane 1) SK-MEL-31 (lane 2), WM 266-4 (lane 3), T98G (lane 4), BV173 (lane 5), CEM (lane 6), MOLT-4 (lane 7), A-427 (lane 8), and SK-MES-1 (lane 9). Lane 10 has no template DNA. Lane 11 shows DNA markers. **c**, Southern blot analysis of melanoma cell lines hybridized to probes 2a2 and *CDK4I5'*. In *Sfi*I-digested pulsed-field blots, 2a2 hybridized to a 54-kb fragment. *CDK4I5'* hybridized to a 190-kb fragment which was

also detected with the 3.3B probe in the same blot (data not shown). Lanes: 1, human placenta; 2, Hs294T; 3, HT144; 4, RPMI7951; 5, SK-MEL-1; 6, SK-MEL-2; 7, SK-MEL-3; 8, SK-MEL-28; 9, SK-MEL-31; 10, WM 266-4.

METHODS. **a**, Phage DNA of clone 10B1 was digested with *Eco*RI and subcloned into *Eco*RI-cut pBluescriptII SK(+) (Stratagene). Subclones were subjected to automated DNA sequencing. The 4.2-kb subclone 10B1-10 contained both the *CDK4I* and the *CDK4I3'* sequences. **b**, PCR amplification was carried out as described in Table 1 legend except that 35 cycles were performed and annealing was at 64 °C and extension at 72 °C. PCR was followed by gel electrophoresis. **c**, DNAs from human placenta and melanoma cell lines were digested with *Eco*RI, resolved on a 0.8% agarose gel, and transferred to nylon membranes. 2a2 is a 1.3-kb *Eco*RI-*Xho*I fragment of phage clone 2a. *CDK4I5'* is a 139-bp product generated by RT-PCR. cDNA from cell line H661 was amplified by PCR using a sense primer (5'-AATTTCGGCAGGAGGAGCAT-3') and an antisense primer (5'-TTATTTGAGCTTTGGTTCTG-3'). PCR products were subcloned and sequenced. Clone p7-4 contained the 5' sequence of CDK4-inhibitor cDNA. A 139-bp product was amplified from clone p7-4 with a sense primer and a new antisense primer (5'-TCGGCTCCGACCGTAAC-3') and used for Southern blotting. Blots were hybridized at 65 °C overnight, washed at 65 °C in 0.1 × SSC containing 0.1% SDS, and exposed to X-ray film. After stripping off radioactivity, the same membrane was rehybridized with probe 2a2.

FIG. 3 Reverse transcriptase-PCR amplification of CDK4-inhibitor mRNA from human malignant cells. The glyceraldehyde 3-phosphate dehydrogenase gene (*G3PDH*) mRNA served as a control. WI-L2 in lane 1 is a normal lymphoblastoid cell line. U937 in lane 2 is a leukaemia cell line. T98G in lane 3 is a glioma cell line. H661 in lane 4, A-549 in lane 5, and SK-MES-1 in lane 6 are non-small-cell lung cancer cell lines. Lane M shows DNA markers. STS-PCR analysis confirmed the presence of STS *CDK4I* in WI-L2, U937, and H661 (data not shown). T98G and SK-MES-1 have the 9p21 deletions shown in Fig. 1a. The deletion in cell line A-549 spans STS *MTAP* to *71F* (data not shown). *CDK4*-inhibitor mRNA was not detected in cell lines with 9p21 deletions that included the *CDK4*-inhibitor gene.

METHODS. mRNA was purified with a FastTrack kit (Invitrogen) and was treated with RNase-free DNase I (Pharmacia) using human placenta DNA as a control to ensure complete digestion by DNase I. After first-strand cDNA synthesis with a Stratascript RT-PCR kit (Stratagene), cDNA was amplified with the *CDK4I3'* primers shown in lower-case in Fig. 2a (58 °C annealing and 70 °C extension). Primers for the *G3PDH* gene (5'-TGGTATCGTGAAGGACTCATGAC-3' and 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3') amplified a 190-bp product (55 °C annealing and 72 °C



extension). RT-PCRs for *CDK4I3'* and *G3PDH* were done separately and resolved on a 2% agarose gel by loading onto the same lanes. As no product was amplified in DNase I-treated human placenta DNA with the *CDK4I3'* primers, the 355-bp RT-PCR product seen in lanes 1, 2 and 4 was derived from cDNA. Sequence analysis of the RT-PCR products confirmed the *CDK4I* cDNA sequence.

placental DNA, after *Eco*RI digestion, and hybridization to the 5'-region probe from the CDK4-inhibitor gene. The most informative cell line was Detroit 562 (a pharyngeal carcinoma) which has a 29-kb deletion within the CDK4-inhibitor gene (Fig. 1). It was positive for STS *CDK4I3'*, negative for STS *CDK4I*, but positive for STS *5BS* and STS *7IF*. The last two STSs are centromeric to the 5' end of the CDK4-inhibitor gene.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assays revealed CDK4-inhibitor gene transcripts in normal cells, but not in cancers with established deletions of the CDK4-inhibitor gene (Fig. 3). Collectively, these results indicate that human cells contain a single CDK4-inhibitor gene, which is homozygously deleted or rearranged in the majority of melanomas, gliomas and leukaemias, and in almost one-third of non-small-cell lung cancers.

The complexes formed by CDK4 and the D-type cyclins control passage through the G1 phase of the cell cycle⁶. The inhibitor of CDK4 is a protein of relative molecular mass 16K which binds to and inhibits the catalytic activity of the CDK4/cyclin D enzymes. Because it is a negative regulator of cell-cycle progression, the inhibitor of CDK4 may be inactivated during cancer development¹⁰. The product of the p53 gene also inhibits growth by stimulating the production of a CDK-inhibitory protein^{11,12}.

About 10% of melanomas are familial². Linkage analyses have pinpointed the locus for familial melanoma to the region on 9p21 that is deleted in sporadic melanomas¹³. Sequence analysis of the CDK4I coding region of a lymphoblastoid cell line (GM06921) derived from a patient with dysplastic nevus syndrome (familial melanoma) showed a C to T transition at position 166 of the mRNA⁶ resulting in a nonsense mutation. Thus the CDK4 inhibitor is a strong candidate for the melanoma susceptibility gene. □

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Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus

Alfredo Lorenzo*, Bronwyn Razzaboni*, Gordon C. Weir† & Bruce A. Yankner*‡

* Department of Neurology, Harvard Medical School and The Children's Hospital, Enders 260, 300 Longwood Avenue, Boston, Massachusetts 02115, USA

† Joslin Diabetes Center and Harvard Medical School, Boston, Massachusetts 02215, USA

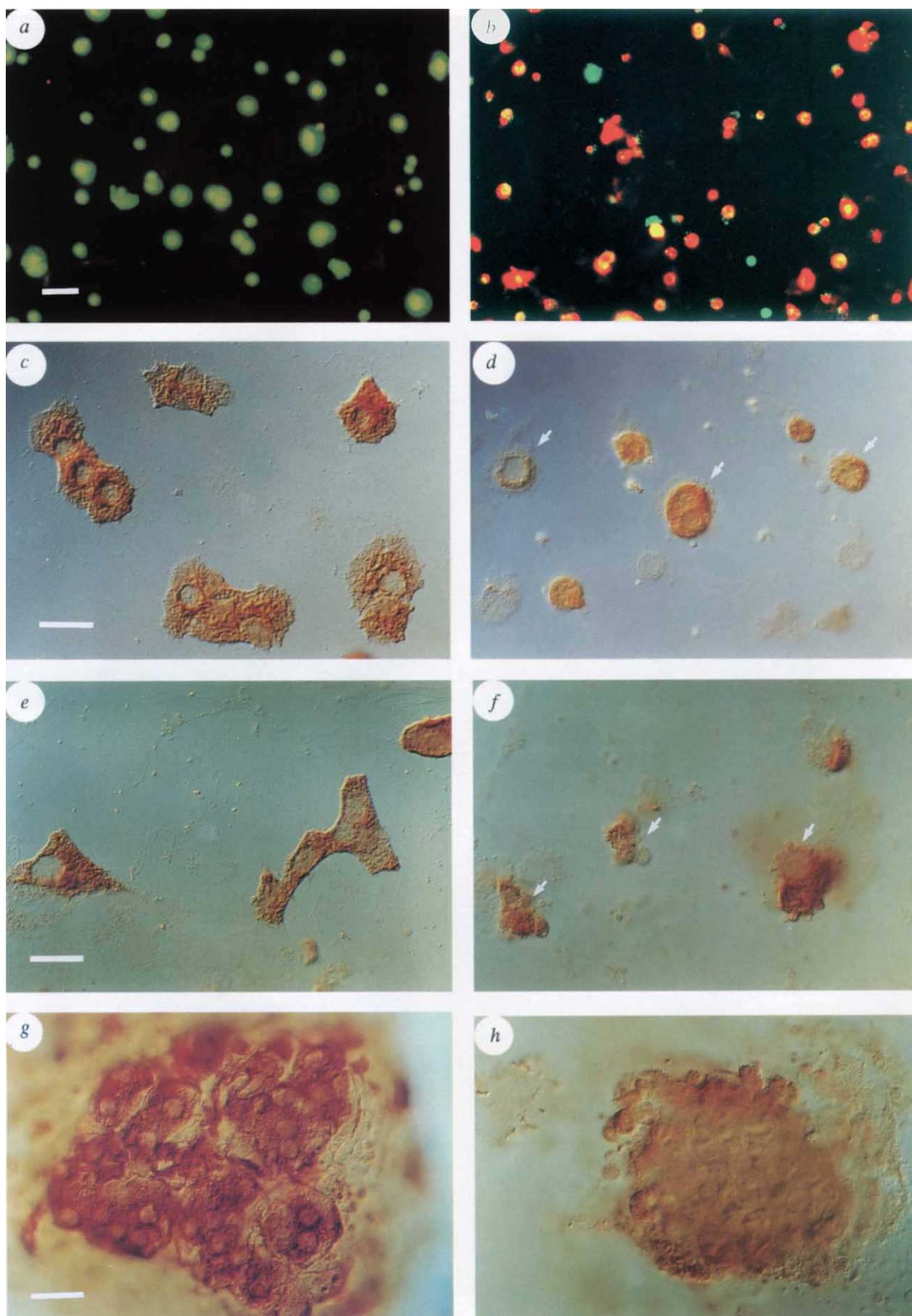
THE 37-amino-acid polypeptide amylin is the principal constituent of the amyloid deposits that form in the islets of Langerhans in patients with type-2 diabetes mellitus^{1–5}, but its role in the pathogenesis of this disease is unresolved^{6–8}. In view of the fact that the β -amyloid protein that forms fibrils in Alzheimer's disease is toxic to neurons^{9,10}, we have investigated whether amylin fibrils could be toxic to pancreatic islet cells. We show here that human amylin is toxic to insulin-producing β -cells of the adult pancreas of rats and humans. This toxicity is mediated by the fibrillar form of the amylin peptide and requires direct contact of the fibrils with the cell surface. The mechanism of cell death involves RNA and protein synthesis and is characterized by plasma membrane blebbing, chromatin condensation and DNA fragmentation, indicating that amylin induces islet cell apoptosis. These findings indicate that amylin fibril formation in the pancreas may cause islet cell dysfunction and death in type-2 diabetes mellitus.

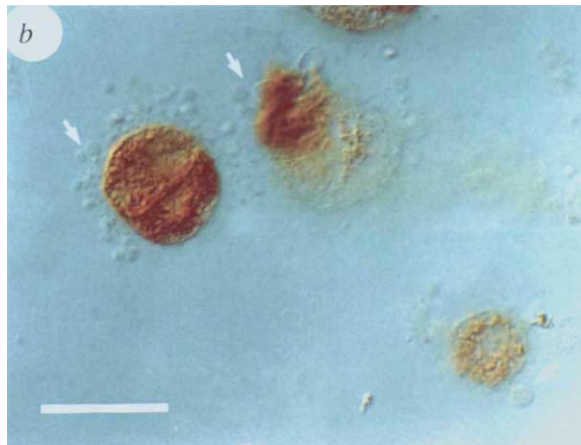
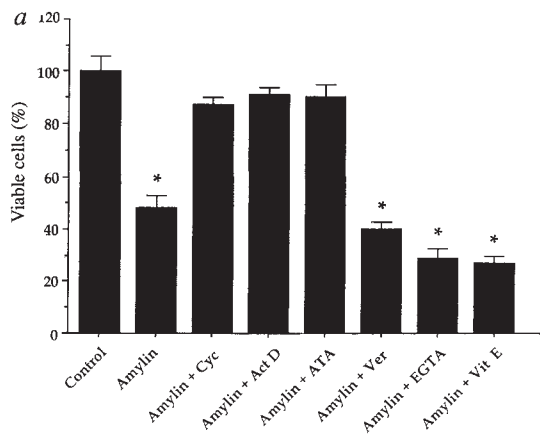
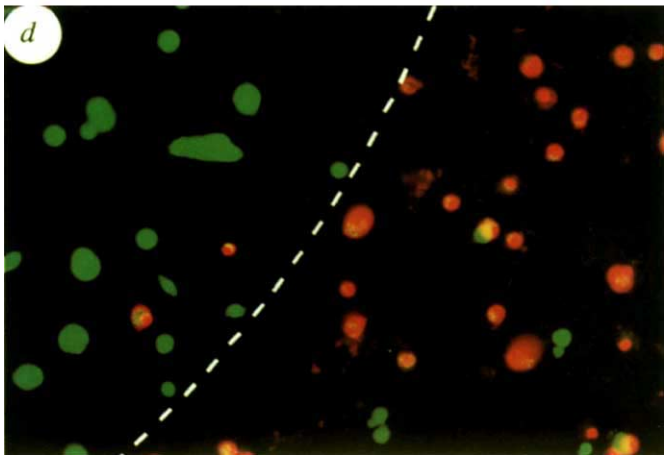
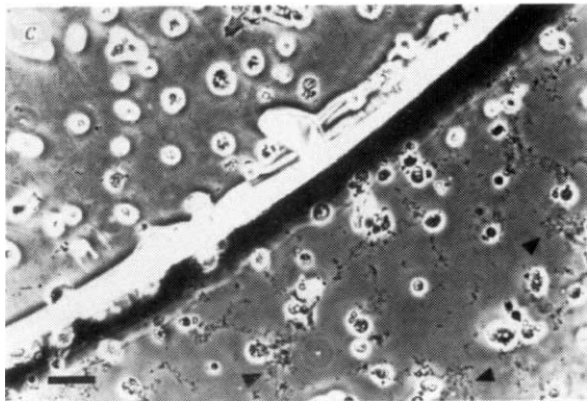
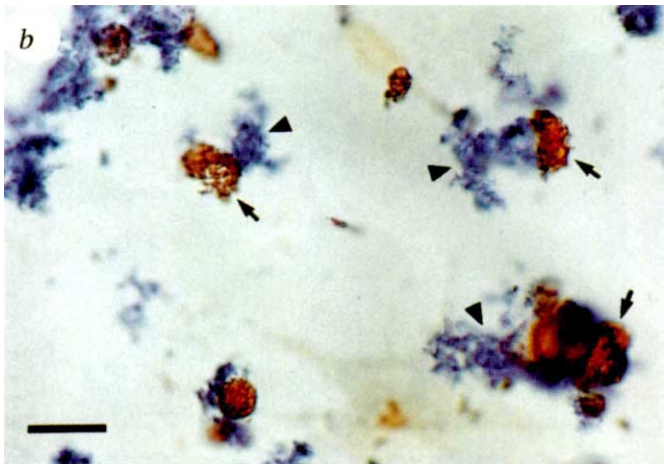
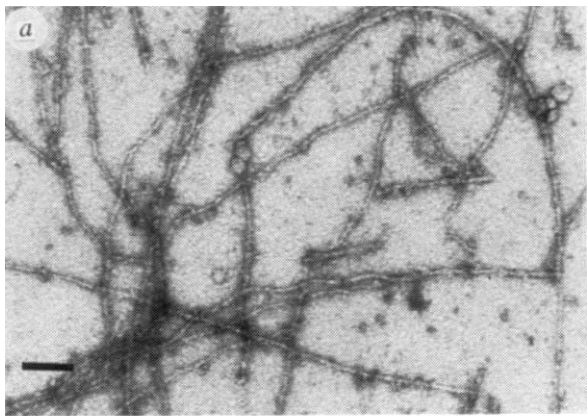
Islet cell cultures were established from adult rat pancreas and cell viability was assessed by a double-fluorescence assay. Exposure to human amylin resulted in substantial rat islet cell death after 24 h (Fig. 1a, b). Immunocytochemical analysis showed that human amylin caused the degeneration of $97 \pm 1\%$ (mean $\pm$ s.e.m., $n = 15$; $P < 0.001$ by ANOVA) of insulin-producing islet cells (Fig. 1c, d). Amylin was also toxic to human islet cells isolated from the pancreas of a 39-year-old woman. Incubation of dissociated human islets with human amylin resulted in the degeneration of $91 \pm 2\%$ ($n = 15$; $P < 0.001$) of insulin-producing islet cells (Fig. 1e, f). Incubation of non-disso-

FIG. 1 Toxicity of human amylin for rat and human pancreatic islet cells. a, Control rat islet cells treated with vehicle and then incubated with calcein-AM and propidium iodide. Epifluorescence microscopy shows viable cells which convert the calcein-AM substrate to a green fluorescent product. b, Rat islet cells after treatment with 20 μ M human amylin for 24 h. There are many dead cells which show red fluorescence due to nuclear uptake of propidium iodide and few viable cells which show green calcein fluorescence. Insulin immunocytochemistry of control (c) and human amylin-treated rat islet (d) cells; e, control, and f, human amylin-treated human islet cells; g, control, and h, human amylin-treated, non-dissociated human islets. Note the degeneration of insulin-positive islet cells after exposure to human amylin (arrows). Scale bars: a, 40 μ m; c, e and g, 20 μ m.

METHODS. Islet cell cultures were maintained for 3–4 days (>90% viability) and then treated with vehicle or 20 μ M human amylin for 24 h. Pancreatic islets were isolated by perfusion of the rat pancreas (from adult male Sprague-Dawley rats weighing 180–200 g) with collagenase²⁷. Human islets were isolated from the pancreas of a 39-year-old woman as described²⁸. Isolated islets were dissociated by incubation in trypsin-EDTA for 10 min at 37 °C and repetitive trituration with a Pasteur pipette. Dissociated islet cells or non-dissociated islets were cultured in 16-mm tissue-culture wells on glass coverslips coated with Matrigel (Collaborative Biomedical Products) in 300 μ l per well of RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin. Lyophilized peptides were dissolved in water to 346 μ M and immediately added to the culture medium at the indicated concentration. An equal volume of sterile water was added to control cultures. Cell viability was determined by the calcein-AM/propidium iodide double-staining method (Molecular Probes)²⁹. Culture medium was removed after treatment and cells were incubated with 1 μ M calcein-AM and 10 μ g ml⁻¹ propidium iodide in phosphate-buffered saline (PBS) for 10 min at 37 °C. Viable cells cleave calcein-AM to calcein, producing uniform green fluorescence; dead cells take up propidium iodide into the nucleus, resulting in intense red fluorescence. Green and red fluorescence were simultaneously visualized using a Nikon transmission microscope equipped with a double-pass filter. For immunocytochemistry, cultures were fixed in 4% paraformaldehyde, 0.12 M sucrose in PBS, pre-incubated with swine serum and stained with a polyclonal primary antibody against insulin using the DAKO PAP kit. Fixed sections of pancreatic tissue were used as a positive control. The viability of insulin-positive cells was determined by propidium iodide staining and scored as described in Table 1 legend. HPLC-purified human, rat and cat amylin (full-length 37-amino-acid peptides) were obtained from Bachem California and Peninsula Laboratories. Human amylin from both sources gave the same results. Peptides were analysed by laser desorption mass spectroscopy (amylin M_r values: human $3,903 \pm 2$ (mean $\pm$ s.e.m., $n = 8$; predicted M_r , 3,904); cat $3,910 \pm 1$ ($n = 3$; predicted M_r , 3,909); rat $3,919 \pm 2$ ($n = 4$, predicted M_r , 3,921)).

‡ To whom correspondence should be addressed.





ciated human islets with human amylin also resulted in toxicity with degenerative morphological features, loss of insulin immunoreactivity and abnormal staining with propidium iodide (Fig. 1g, h). Non-pancreatic cell types showed differential vulnerability to amylin toxicity. Human amylin was toxic to primary rat hippocampal neurons¹¹, primary aortic endothelial cells, COS cells and PC12 pheochromocytoma cells, whereas primary rat and human fibroblasts and H4 hepatoma cells were resistant (data not shown).

Islet cell toxicity was first detected at a concentration of 5 μ M human amylin and almost complete islet cell death occurred at 20 μ M amylin in both rat and human islet cell cultures (Table 1). The formation of amylin fibrils in a crossed β -pleated sheet conformation was assessed by Congo red birefringence under polarized light¹² and by electron microscopy (Table 1; Fig. 2a); formation of amylin fibrils showed a concentration dependence which paralleled the concentration dependence of human amylin toxicity (Table 1).

◀ FIG. 2 The amylin fibril is toxic. a, Electron micrograph of amylin aggregates formed in islet cell culture. Human amylin (20 μ M) forms long, non-branched fibrils of 6–9 nm diameter. b, Nomarsky microscopy of rat islet cells treated with human amylin and double-labelled for amylin (blue) and tubulin (brown). Amylin aggregates (arrowheads) are closely associated with degenerating islet cells (arrows). c and d, Effects of human amylin on an islet cell culture partially covered by a glass coverslip. The coverslip covers cells to the left of the edge in c and to the left of the dotted line in d. c, Phase contrast microscopy after treatment with 20 μ M human amylin for 24 h shows amylin aggregates (arrowheads) associated with cells outside the coverslip but not under the coverslip. d, Epifluorescence microscopy after treatment with human amylin and staining with calcein-AM and propidium iodide shows viable cells (green) under the coverslip and dead cells (red) outside the coverslip. Scale bars: a, 100 nm; b, 20 μ m; c, 40 μ m.

METHODS. Electron microscopy of negatively stained amylin is described in Table 1 legend. The coverslip protection experiment was done by growing islet cells on a 12-mm matrigel-coated coverslip partially covered with a 5-mm coverslip. Double-label immunocytochemistry for amylin and tubulin was achieved by incubation with polyclonal rabbit anti-human amylin (Peninsula Labs) and monoclonal anti- α -tubulin (clone DM1A; Sigma) followed by alkaline-phosphatase-conjugated anti-rabbit IgG, biotin-conjugated anti-mouse IgG and Extravidin-peroxidase (Sigma). Cultures were then reacted with diaminobenzidine and nitroblue tetrazolium.

◀ FIG. 3 Human amylin induces islet cell apoptosis. a, Effects of different agents on the islet cell toxicity of human amylin. Islet cells were treated with 8 μ M human amylin for 24 h in the absence or presence of 10 μ M cycloheximide (Cyc), 5 nM actinomycin D (Act D), 100 μ M aurintricarboxylic acid (ATA), 10 μ M verapamil (Ver), 10 mM EGTA and 100 μ g ml⁻¹ α -tocopherol (Vit E). These agents did not produce significant changes in cell viability when tested alone. Values are the mean $\pm$ s.e.m., $n = 15$, from a representative experiment. The effect of each agent was replicated in at least three separate experiments. * $P < 0.001$ relative to the control by analysis of variance (ANOVA) with post hoc Tukey test. b, Prominent membrane blebbing (arrows) after treatment of islet cells with human amylin. Shown are islet cells immunocytochemically stained for insulin and viewed with Nomarsky optics. Scale bar, 20 μ m. c, Chromatin condensation characteristic of apoptotic cells after treatment of islet cells with human amylin. Epifluorescence microscopy after chromatin staining with propidium iodide. d, DNA fragmentation after treatment with human amylin. Agarose gel is shown of islet cell DNA after incubation for 24 h in a control culture (lane 2), with 15 μ M rat amylin (lane 3), with 15 μ M human amylin (lane 4), and after incubation for 48 h in a control culture (lane 5) and with 15 μ M human amylin (lane 6). DNA size markers of 0.3–12 kb are shown in lanes 1 and 7.

METHODS. DNA fragmentation was assessed in primary rat islet cells. Islet cells were lysed by suspension in 1% SDS, 10 mM EDTA, 0.4 M NaCl and 10 mM Tris-HCl, pH 8.2, and incubated overnight with 0.2 mg ml⁻¹ proteinase K at 55 °C. DNA was extracted in phenol/chloroform, precipitated in 0.3 M sodium acetate in 100% ethanol, resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and incubated with DNase-free RNase (20 μ g ml⁻¹ for 2 h). DNA was resolved by electrophoresis in a 1% agarose gel and stained with ethidium bromide.

We also examined the toxicity of amylin from different species with different fibril-forming capacities. Cat amylin differs from human amylin at only four amino acids and forms islet amyloid deposits *in vivo*^{3,4,13}; this amylin caused extensive islet cell death but at a higher concentration than human amylin, which paralleled the concentration dependence of fibril formation (Table 1). Rat amylin is also highly homologous to human amylin but differs at six amino acids, resulting in a non-amyloidogenic peptide^{13–16}. Rat amylin was not toxic to islet cells and did not form fibrils (Table 1). Thus, the toxic potency of amylin correlates with the intrinsic capacity of the peptide to form amyloid fibrils.

We next determined whether the physical association of amylin fibrils with islet cells was necessary for toxicity. Electron microscopy of insoluble amylin aggregates concentrated by centrifugation of culture medium showed that amylin fibrils were present (Fig. 2a). Exposure of islet cells to human amylin followed by immunocytochemical staining demonstrated that amylin aggregates were clearly associated with degenerating islet cells

TABLE 1 Concentration dependence of amylin toxicity and fibril formation

Peptide	Viable cells (%)		Congo red	Fibrils
	Rat islets	Human islets		
Control	100 $\pm$ 7	100 $\pm$ 2	–	–
Human amylin (μ M)				
0.1	98 $\pm$ 6		–	–
0.5	98 $\pm$ 5		–	–
1	103 $\pm$ 6	103 $\pm$ 4	–	–
5	66 $\pm$ 4*	88 $\pm$ 2*	+	+
10	35 $\pm$ 4**	48 $\pm$ 3**	+	+
20	5 $\pm$ 1**	9 $\pm$ 2**	+	+
40	0.4 $\pm$ 0.4**	2 $\pm$ 1**	+	+
Cat amylin (μ M)				
1	103 $\pm$ 8		–	–
5	100 $\pm$ 6		–	–
10	105 $\pm$ 6		–	–
20	88 $\pm$ 6		–	–
40	39 $\pm$ 4**	42 $\pm$ 7**	+	+
Rat amylin (μ M)				
1	98 $\pm$ 5		–	–
10	107 $\pm$ 8		–	–
20	98 $\pm$ 6		–	–
40	99 $\pm$ 6	100 $\pm$ 1	–	–
β -Amyloid(25–35) (μ M)				
1	98 $\pm$ 4		–	–
10	87 $\pm$ 3		+	+
20	76 $\pm$ 5*		+	+
40	66 $\pm$ 4**		+	+

Rat and human islet cells were incubated with the indicated concentration of each amylin for 24 h and with β -amyloid (25–35) for 48 h. Islet cell viability was determined as described in Fig. 1 legend by propidium iodide uptake for rat islet cells, and a combination of propidium iodide staining and insulin immunocytochemical staining for human islet cells. Cell viability is expressed as the percentage of viable cells relative to the number of cells in control cultures. Values are means $\pm$ s.e.m., $n = 15$. Five 0.8-mm² fields were scored per well in triplicate 16-mm wells. At least 300 islet cells were scored for each condition. For Congo red staining, 0.3 ml culture medium containing each peptide at the indicated concentration was centrifuged at 90,000g for 1 h. The pellet containing insoluble peptide was air-dried, stained with 1% Congo red solution and viewed under polarized light. Electron microscopic evaluation of fibril formation was made on the insoluble peptide concentrated by centrifugation of cell-free culture medium and negatively stained with 1% uranyl acetate. The presence (+) or absence (–) of fibrils (Fig. 2a) was determined at 38,000 $\times$ magnification. β -Amyloid(25–35) peptide (Bachem) was dissolved in sterile water and added directly to the culture medium.

* $P < 0.05$; ** $P < 0.001$ relative to the control by analysis of variance (ANOVA) with post hoc analysis by the Tukey test.

(Fig. 2b). Islet cells were then treated in a culture in which some cells were protected in a sandwich between two glass coverslips while adjacent cells were exposed. Amylin aggregates formed in association with islet cells outside the coverslip but not inside the coverslip sandwich (Fig. 2c). There was a toxic effect outside the sandwich where islet cells were in contact with amylin aggregates, but islet cells inside the sandwich remained viable (Fig. 2d). To confirm that islet cells inside the coverslips were accessible to soluble peptide, cultures were treated with a high concentration of the soluble toxic peptide mastoparan, which resulted in islet cell degeneration both inside and outside the coverslip (data not shown). These results suggest that insoluble fibrillar amylin is the toxic agent.

We also examined the effects of an unrelated amyloidogenic peptide, the β -amyloid protein. The neurotoxic β (25–35) homologue of β -amyloid¹⁰ was toxic to islet cells in the amyloidogenic concentration range (Table 1). A similar result was obtained for the aggregated β (1–40) homologue of β -amyloid (data not shown). The toxic potency of the amyloidogenic β -amyloid peptides was much less than that of human amylin (Table 1). Amyloid fibrils are therefore generally toxic to islet cells, but their toxic potency is peptide-specific.

We then investigated whether amylin-induced islet cell degeneration was occurring by a mechanism of programmed cell death. The protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D both inhibited amylin islet cell toxicity (Fig. 3a). Aurintricarboxylic acid, an endonuclease inhibitor that prevents death in cells undergoing apoptosis¹⁷, almost completely inhibited amylin islet cell toxicity (Fig. 3a). Inhibition of calcium influx with the calcium-channel blocker verapamil, or with the calcium-chelator EGTA, had no significant effect (Fig. 3a). Morphological changes characteristic of apoptosis include the formation of membrane spheroids or blebs, chromatin condensation and nuclear fragmentation¹⁸. Islet cells treated with amylin exhibited prominent membrane blebbing (Fig. 3b), and nuclear staining with propidium iodide showed that chromatin was condensed (Fig. 3c). Nuclear DNA from amylin-treated islet cells was fragmented (Fig. 3d). DNA oligomers that were multiples of ~180–200 base pairs were resolved, consistent with internucleosomal DNA cleavage. These results indicate that fibrillar amylin peptide induces apoptosis of pancreatic islet cells.

Our experiments show that fibrillar amylin is toxic to insulin-producing β -cells of the rat and human pancreas, indicating that amylin fibril formation may be important in the pathogenesis of type-2 diabetes. Deposits of amylin fibrils can occupy much of the pancreas and are associated with islet cell loss in type-2 diabetes^{7,8,19,20}. Islet amyloid deposition is species-specific and is associated with a maturity-onset diabetic syndrome in humans and cats, but not in most rodents^{13–16}, correlating with the toxicity of amylin polypeptides from these species (Table 1).

The toxic effect of fibrillar amylin is strikingly similar to that of the β -amyloid protein^{9–11} and of a peptide from the prion protein²², which form amyloid fibrils in Alzheimer's disease²¹ and the spongiform encephalopathies²³, respectively. Like amylin, β -amyloid and the prion-protein-derived peptide induce apoptosis of target cells^{22,24–26}. These observations suggest that the formation of toxic amyloid fibrils may cause programmed cell death in several human degenerative disorders. □

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Calcium/calmodulin inhibition of basic-helix-loop-helix transcription factor domains

Brit Corneliussen, Magnus Holm, Yvonne Waltersson*, Jacqueline Onions, Bengt Hallberg*, Anders Thornell* & Thomas Grundström†

Department of Applied Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden

THE ubiquitous Ca^{2+} -binding protein calmodulin (CaM) is a key protein in Ca^{2+} homeostasis and activation of eukaryotic cells. CaM is the molecular link between free Ca^{2+} in the cell and the inhibition, or activation, of numerous enzymes. Many nuclear functions are under Ca^{2+} /CaM control, and some transcriptional activators are known to be Ca^{2+} modulated indirectly through Ca^{2+} /CaM-dependent protein kinases^{1–4}. But Ca^{2+} /CaM has not yet been found to directly modulate any transcription factor or other DNA-binding protein. Transcription factors of the basic-helix-loop-helix (bHLH) group are important regulators in numerous systems^{5–11}. Here we report that binding of Ca^{2+} -loaded CaM to the bHLH domains of several bHLH proteins directly inhibits their DNA binding. Other bHLH proteins are either less sensitive or resistant. Ca^{2+} ionophore selectively inhibits transcriptional activation by Ca^{2+} /CaM-sensitive bHLH proteins *in vivo*, implying that Ca^{2+} can directly influence transcription through differential CaM inhibition of bHLH domains.

On the basis of preliminary studies identifying DNA-binding proteins interacting with CaM, we purified these proteins from calf thymus nuclei using a CaM-Sepharose column. The subsequent final column contained an E-box DNA sequence, which binds bHLH proteins^{3,11}, and eluted proteins were designated ECa (E-box and Ca^{2+} /CaM binding). The ECa proteins produced sequence-specific retarded complexes with the E-box of a DNA probe in electrophoretic mobility shift assays (EMSA) (Fig. 1a, lanes 2–4, and data not shown). Figure 1a shows inhibition of E-box binding of ECa by addition of Ca^{2+} /CaM (lanes 5–9). Inhibition was obtained at 25 nM CaM, and at 500 nM Ca^{2+} , which is far below the nuclear CaM concentration^{12–14}, the inhibition exceeded 10-fold. Chelation of Ca^{2+} blocked

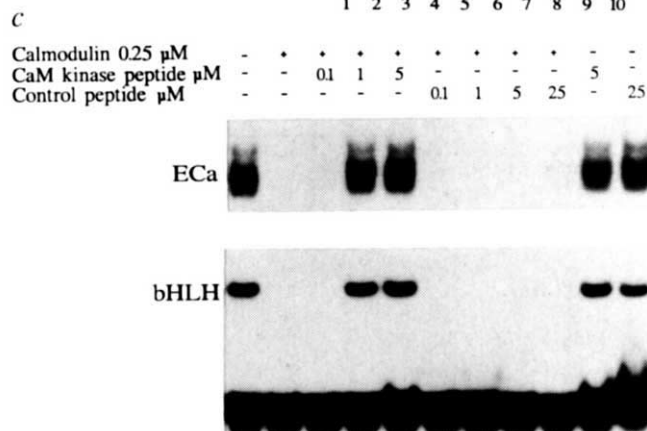
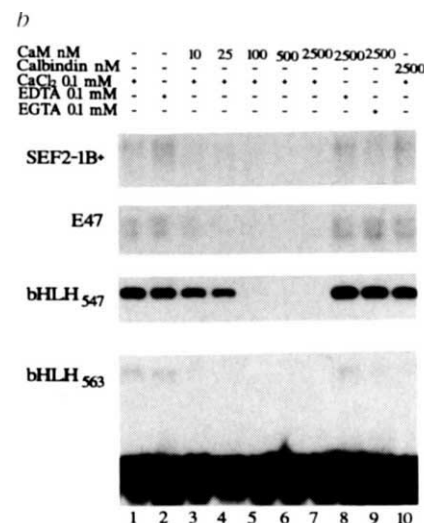
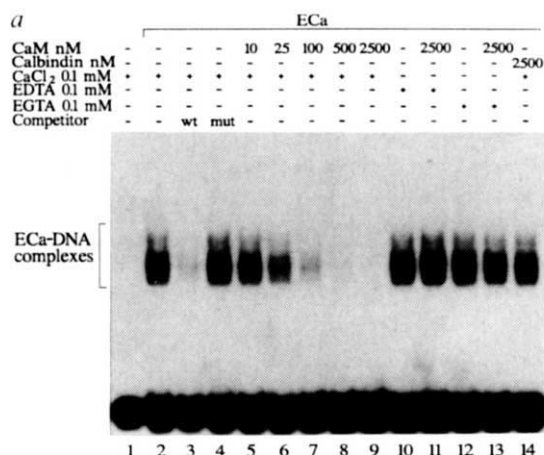
* Present addresses: Department of Clinical Chemistry, Malmö hospital, Malmö, Sweden (Y.W.); Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK (B.H. and A.T.).
† To whom correspondence should be addressed.

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FIG. 1 Analysis of CaM inhibition of E-box binding in EMSA. An end-labelled DNA segment (2 fmol) containing the E-box of the SL3-3 virus enhancer (5'-CCAAGAACAG-ATGGTCCCC-3') was used. Where indicated, CaM, calbindin D_{9k}²⁰, CaCl₂, EDTA or EGTA was added. **a**, CaM inhibition of 2 ng purified ECa (E-box and CaM binding) proteins. Lanes 3 and 4 are with 50-fold excess of unlabelled wild-type and point mutated (5'-CCAAG-AAAC7CTGGTCCCC-3') E-box, respectively. **b**, CaM inhibition of SEF2-1B+, E47, and the bHLH domain (amino acids 547–622 and 563–622⁵) of SEF2-1. **c**, Effects of peptides on CaM inhibition. Where indicated, a known CaM-binding peptide¹⁵, amino acids 290–309 of CaM kinase II, or another basic peptide, CRRRGKGGHDGLYQL, was added 5 min before ECa or bHLH₅₄₇. All reactions contained 0.1 mM CaCl₂.

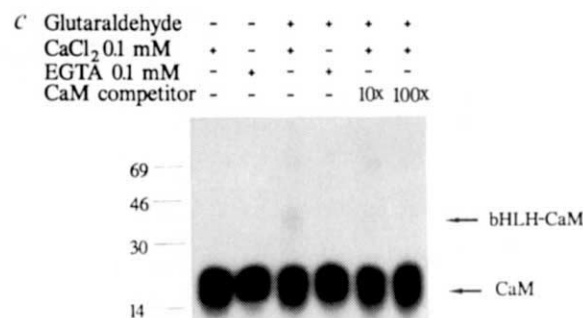
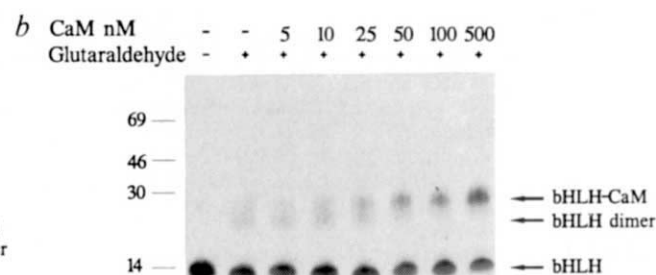
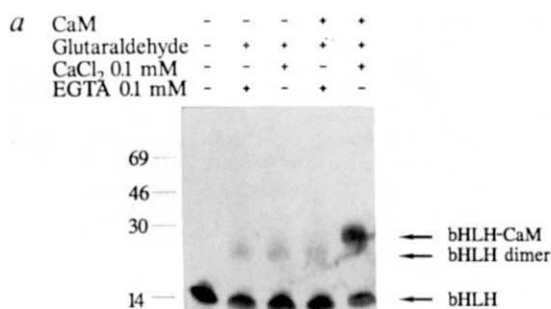
METHODS. For ECa purification, nuclear extracts were prepared from 17 bovine calf thymuses as described²¹ except using pH 5.0 and no EDTA in the final buffer. S-Sepharose, Heparin-Sepharose CL-6B and salmon DNA-Sepharose chromatographies were as described²¹ except omitting EDTA, washing at 0.1 M NaCl and eluting with 0.7 M, 1.0 M and 1.0 M NaCl, respectively. The eluate was diluted to 0.1 M NaCl, 2 mM CaCl₂ and pH 7.3 and applied to a 5 ml CaM-Sepharose 4B column with 25 mg of the CaM of higher vertebrates produced in *Escherichia coli*²². The column was washed at 0.25 M NaCl, 0.2 mM CaCl₂ and eluted by replacing the CaCl₂ with 2 mM EDTA. The final DNA sequence/ Sepharose chromatography was as described²³, using an optimized E-box sequence⁵ (a concatamer of 5'-CCAAGAACAGCTGTCCCC-3'), and yielded 6 µg ECa proteins. CaM-binding reactions were in 10 mM HEPES, pH 7.6, 5% glycerol, 0.025% Triton X-100, 1 mM DTT, 50 mM NaCl, 40 ng µl⁻¹ BSA and either CaCl₂, EDTA or EGTA at room temperature for 5 min. DNA probe was added in 2× EMSA buffer²⁴ without EDTA; EMSAs were as described²⁴. cDNAs were *in vitro* translated using the TnT coupled reticulocyte lysate system (Promega) with pSP64poly(A)⁺ and SP6 RNA polymerase, and proteins were batch-purified with one volume of Heparin-Sepharose as for ECa purification except using 2.5 mM DTT.



SEF2-1B+ cDNA encodes full-length SEF2-1B^o with the four additional amino acids at position 546 that many SEF2-1 cDNAs encode⁵. The mouse E47 cDNA starts from amino-acid 46²⁵ (longer than the A1 clone²⁶).

FIG. 2 Glutaraldehyde cross-linking of CaM and the bHLH domain of SEF2-1. Where indicated, CaM and/or glutaraldehyde was added to the reactions. The positions of bHLH₅₄₇ (see Fig. 1), CaM, cross-linked bHLH₅₄₇ dimer, cross-linked bHLH₅₄₇-CaM and *M_r* standards (×10³) in the SDS-PAGE are indicated. **a**, Crosslinking of ³⁵S-labelled bHLH₅₄₇ with Ca²⁺-loaded CaM. Where indicated, 0.1 mM CaCl₂ or EGTA was added to the reactions. **b**, Crosslinking of ³⁵S-labelled bHLH₅₄₇ at increasing concentrations of CaM in the presence of 0.1 mM CaCl₂. **c**, Crosslinking of ¹²⁵I-labelled Ca²⁺-loaded CaM with bHLH₅₄₇, and competition with unlabelled CaM. The reactions contained 5 nM ¹²⁵I-labelled CaM and either CaCl₂ or EGTA. The excess of CaM competitor over labelled CaM is indicated. The weak upper band represents background crosslinking of CaM to a component(s) of the *in vitro* translation extract.

METHODS. bHLH₅₄₇ (see Fig. 1) was labelled by including 0.8 mCi ml⁻¹ ³⁵S-methionine in the *in vitro* translation (see Fig. 1), and CaM was ¹²⁵I-labelled using the Enzymobead (Bio-Rad) modification of the lactoperoxidase-catalysed iodination technique. Binding reactions were as in Fig. 1 except that no DTT or EDTA was added. Crosslinking used 0.01% glutaraldehyde for 20 min at room temperature, followed by



inactivation with SDS sample buffer. Proteins were analysed by SDS-PAGE (10%).

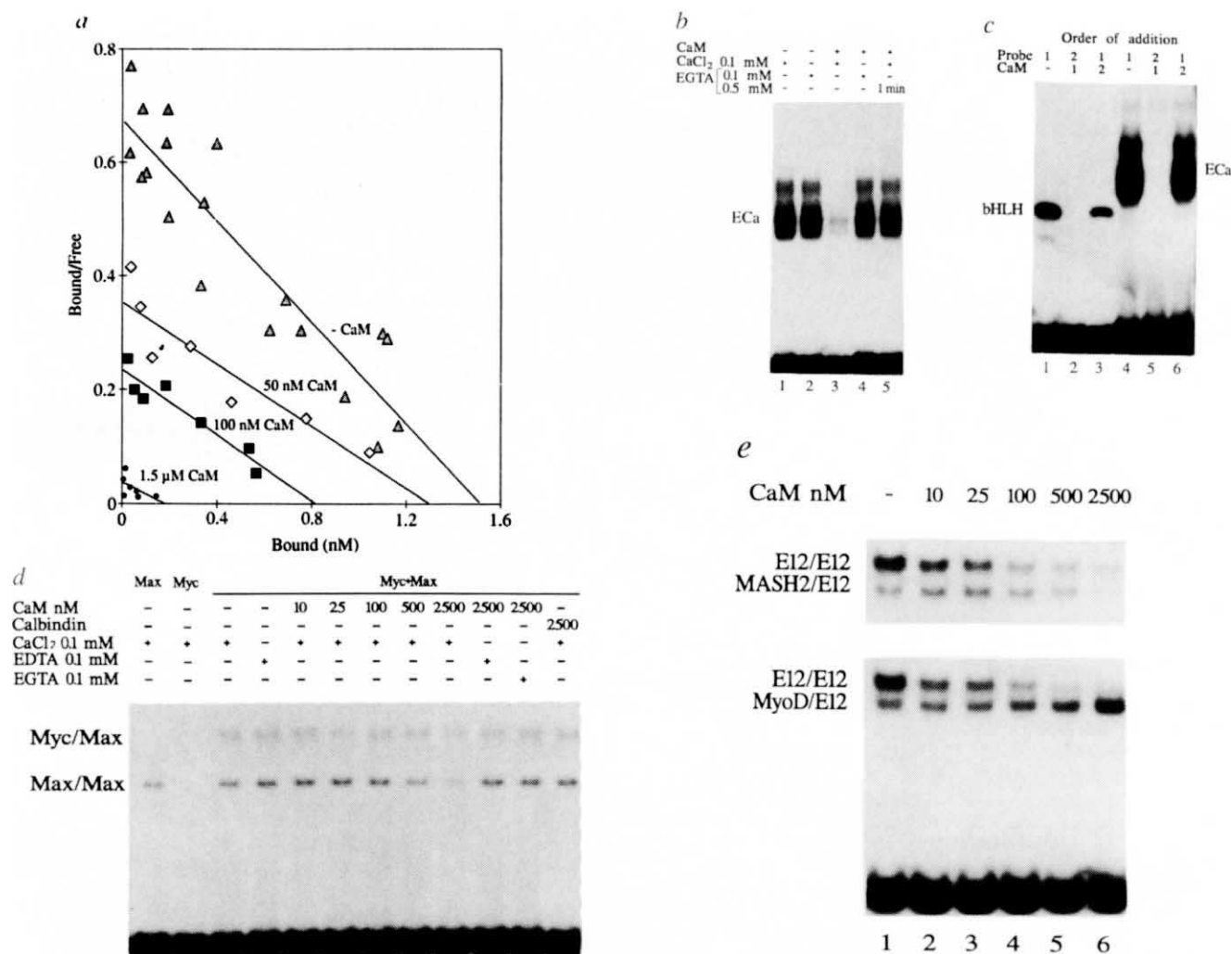


FIG. 3 Characterization of interactions between various E-box-binding proteins and CaM using EMSA. **a**, Scatchard plot analysis of ECa binding to the E-box probe (see Fig. 1) at various CaM concentrations. CaM was added 5 min before the probe and 0.1 mM CaCl₂ was present. Amounts of complexes and free DNA were quantified by scintillation counting of excised gel pieces. **b**, Reversibility of CaM inhibition on EGTA chelation of Ca²⁺ ions. 1 μM CaM and 0.1 mM CaCl₂ or EGTA was added to the binding reactions. In lane 5 the CaCl₂ was chelated with 0.5 mM EGTA 1 min before electrophoresis. **c**, Decreased inhibition of bHLH₅₄₇ and ECa on interaction with the DNA before CaM. The order of addition of probe and CaM (2.5 μM) to bHLH₅₄₇ (lanes 1–3) and ECa (lanes 4–6) in presence of 0.1 mM CaCl₂ is indicated. The incubation time between probe/CaM additions was 5 min. **d**, CaM effect on Max (amino acids 14–94) homo-oligomers and Myc (C-terminal 172 amino acids)/Max

hetero-oligomers. EMSA conditions were as in Fig. 1 except using the DNA sequence 5'-TCAGACCACGTGGTCTGGT-3' as probe. **e**, CaM effect on E12 (C-terminal 169 amino acids) homo-oligomers plus MASH2 (amino acids 98–174)/E12 hetero-oligomers and E12 homo-oligomers plus MyoD (amino acids 90–165)/E12 hetero-oligomers. 0.1 mM CaCl₂ was used in all reactions and the end-labelled DNA segment 5'-AGGCAGCAGGTGTTGGGG-3' from the muscle creatine kinase (MCK) enhancer was used as probe.

METHODS. The cDNAs encoding parts of human Myc plus Max, mouse E12 plus mouse MyoD and E12 plus mouse MASH2 were cotranslated and the synthesized proteins purified as described (Fig. 1). In **e** the probe was added in the same DNA-binding buffer as described¹⁶ without EDTA; EMSA were as described¹⁶ except that EDTA was excluded.

inhibition, and neither calbindin D_{9K}, one of the CaM protein super family, nor bovine serum albumin had any inhibitory effect on the DNA binding of ECa (lanes 11, 13 and 14, and data not shown). Thus, CaM inhibition of E-box binding is Ca²⁺-dependent and specific.

The E-box used in the EMSA can bind bHLH proteins encoded by the SEF2-1⁵ (also named ITF2⁶) and E2A genes (B.C. and T.G., unpublished observations). Therefore we expressed complementary DNAs from these genes in a reticulocyte extract and partially purified the proteins. Figure 1b shows that CaM efficiently inhibited DNA binding of both SEF2-1B+, a full-length human SEF2-1, and a near full-length E47 from the mouse E2A gene. The inhibition was totally Ca²⁺ dependent, and no effect of the closely related calbindin D_{9K} was observed (lanes 8–10). All SEF2-1 deletion mutants that kept the bHLH DNA-binding domain, including the bHLH domain alone, bHLH₅₄₇ (amino acids 547–622⁵), were inhibited by CaM (Fig.

1b and data not shown). Even bHLH₅₆₃, with a partial deletion of the basic region, was sensitive to Ca²⁺/CaM (Fig. 1b). Deleted proteins from E2A that retained the bHLH domain were also inhibited by Ca²⁺/CaM (see below). Thus, Ca²⁺-loaded CaM directly inhibits at least some bHLH domains.

We used a CaM-binding peptide¹⁵ of Ca²⁺/CaM-dependent kinase (CaM kinase) II to analyse the specificity of interaction between CaM and E-box-binding proteins. The CaM kinase peptide completely blocked Ca²⁺/CaM inhibition of both ECa and bHLH₅₄₇, whereas a control peptide with an unrelated basic sequence could not block the inhibition (Fig. 1c). Thus, a peptide with a well characterized CaM-binding domain blocks the CaM inhibition of DNA binding.

To analyse the possibility of direct interaction between CaM and the bHLH domain, we used glutaraldehyde crosslinking followed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE). A band representing CaM

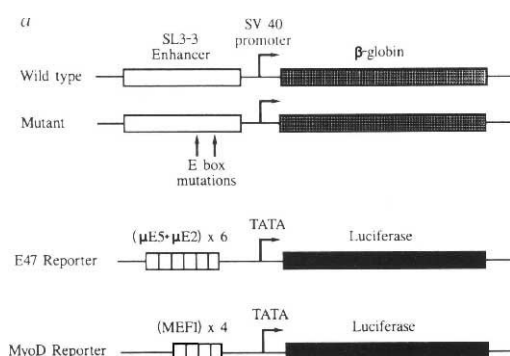
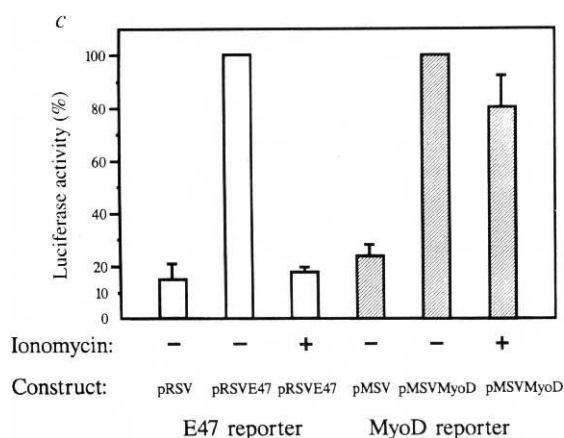
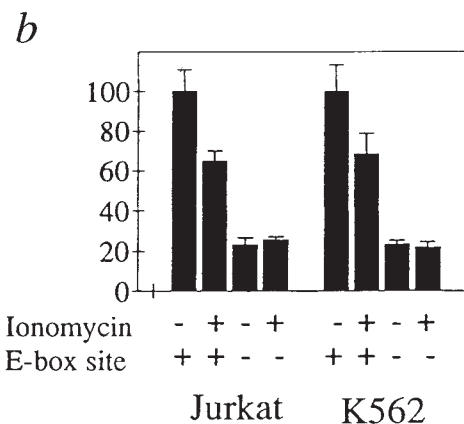


FIG. 4 Effect of the Ca^{2+} ionophore ionomycin on the activity of wild-type and E-box-mutated SL3-3 virus enhancer, and on the activity of transfected E47 and MyoD on reporter constructs, in different cell lines. **a**, Schematic representation of reporter constructs used. The wild-type construct and the corresponding construct with the E-box mutation (Fig. 1a) used in **b** have been described previously^{5,27}. Reporter constructs used in **c** were made by insertion of a hexameric $\mu E5$ - $\mu E2$ site followed by the alkaline phosphatase TATA box⁶ (E47 reporter) and a tetrameric MEF1 site followed by the thymidine kinase TATA box²⁸ (MyoD reporter), respectively, in front of the luciferase gene of pGL2basic (Promega). Cotransfected expression plasmids were with E47 cDNA as in Fig. 1 or without E47 after the RSV promoter/enhancer, MyoD expression vector under control of the MSV promoter/enhancer²⁹ (pEMC11(s)) and corresponding control vector without MyoD cDNA, denoted pRSV-E47, pRSV, pMSV-MyoD and pMSV, respectively. **b**, Average amounts of specific RNA in four transfection experiments using a reference plasmid, pESG53²⁷, (determined by quantitative S1 nuclease mapping²⁷) expressed relative to the wild type without ionomycin added, which was taken as 100%. The lines represent ± 1 s.d. Where indicated, ionomycin ($1 \mu g ml^{-1}$) was added 24 h after transfection, and collections were 42 h after transfection. **c**, Average luciferase activity in 4–10 transfections expressed relative to pRSV-E47 and pMSV-MyoD without ionomycin added, which were taken as 100% for the two reporter plasmids, respectively. The lines represent ± 1 s.d. Where indicated, ionomycin ($0.5 \mu g ml^{-1}$) was added 0.5 h after transfection, and collections were 7.5 h later. Transfections were as described³⁰ with the modification that



5 μg each of expression vector, reporter plasmid and the hCMV- β -gal plasmid (reference plasmid for normalization³⁰) were cotransfected. Luciferase activities were determined with Luciferase Assay System (Promega).

crosslinked to ^{35}S -labelled bHLH₅₄₇ was obtained when both CaM and Ca^{2+} were present (Fig. 2a). Roughly the same concentration of CaM (10 nM) was required for noticeable crosslinking of CaM-bHLH as for noticeable inhibition of E-box binding (compare Figs 1b and 2b). ^{125}I -labelled CaM could also be crosslinked to bHLH₅₄₇ in the presence of Ca^{2+} , and this crosslinking was inhibited by an excess of non-labelled CaM (Fig. 2c). The results demonstrate that the bHLH domain and CaM interact without any observable intermediary molecule. A number of ECa proteins could also be crosslinked to $Ca^{2+}/^{125}I$ -CaM, yielding products with different SDS-PAGE mobilities (data not shown).

Scatchard plot analysis of E-box binding of ECa at various DNA concentrations yielded an apparent dissociation constant of about 2.3 nM (Fig. 3a). Preincubation with increasing concentrations of CaM did not affect the dissociation constant, but rather led to disappearance of active ECa molecules. To ensure that inhibition of DNA binding of ECa by Ca^{2+} /CaM was reversible, we analysed the effect of subsequent chelation of Ca^{2+} . A high degree of reversion was obtained within 1 min (Fig. 3b, lane 5), indicating that CaM reversibly inhibits ECa by making the DNA-binding structure inaccessible. Inhibition of both bHLH₅₄₇ and ECa decreased drastically when the protein was allowed to interact with the DNA before CaM addition (Fig. 3c, lanes 3 and 6). Thus, the bHLH domain becomes inaccessible for CaM inhibition when it is engaged in E-box binding.

Results from ECa purification indicated that many E-box binding factors do not adhere to CaM, and that ECa also con-

tained bHLH proteins other than SEF2-1 and E12/E47 (data not shown). We therefore analysed effects of CaM on additional bHLH proteins. The DNA-binding domains of the oncoprotein Myc plus its heterooligomer partner Max¹⁰, E12 plus the myogenic protein MyoD, and E12 plus mammalian achaete-scute homologue 2 (MASH2) were cotranslated. The E12 and Myc cDNAs also encoded additional sequences enabling distinction of homodimer and heterodimer complexes in EMSA (Fig. 3d, e). Max homo-oligomers were inhibited by CaM although they showed a much lower sensitivity than E12/E47 or SEF2-1, whereas Myc/Max hetero-oligomers did not display CaM inhibition (Fig. 3d). The inhibition of Max homo-oligomers was Ca^{2+} -dependent and specific (Fig. 3d). Coexpressions of MASH2 or MyoD with E12 resulted in poor formation of MASH2 or MyoD homodimer complexes as previously reported^{16,17}. Much higher CaM concentrations were needed for inhibition of MASH2/E12 hetero-oligomers than for E12 homo-oligomers (Fig. 3e). The most remarkable results were obtained with MyoD, which acts in myogenesis as a heterodimer with E12/E47 or a related protein⁷. In extreme contrast to E12 homo-oligomers, MyoD/E12 hetero-oligomers were totally resistant to CaM (Fig. 3e). Glutaraldehyde crosslinking resulted in much less CaM crosslinked product for MyoD than for the other studied bHLH proteins in accordance with the results of the DNA-binding analyses (data not shown).

Lower CaM sensitivity of several hetero-oligomers compared to a corresponding homooligomer was not a general property of bHLH proteins, because SEF2-1/E12 hetero-oligomers were

as CaM sensitive as the homo-oligomers (data not shown). The tested bHLH proteins with high CaM sensitivity belong to class A¹⁸. These proteins are broadly expressed and can hetero-oligomerize with more cell-type-restricted bHLH proteins of class B¹⁸. The differential CaM sensitivities may therefore provide a Ca²⁺-dependent mechanism for selectively favouring formation of the functionally important hetero-oligomers between class A and class B proteins.

In vivo, addition of a Ca²⁺ ionophore, ionomycin, could clearly decrease the activity of the E-box containing SL3-3 murine leukaemia virus enhancer in both Jurkat and K562 cells (Fig. 4a, b). The activating effect of the E-box, but not a point-mutated derivative, diminished with ionomycin, as if the Ca²⁺ signal inhibits E-box binding transcriptional activators. Ca²⁺-dependent modulation of transcriptional activation by transiently expressed E47 was analysed using a luciferase μ E5 + μ E2 E-box reporter plasmid (Fig. 4a) in the DG-75 Burkitt's lymphoma. The normalized activation of luciferase expression in the presence of the cotransfected E47 gene almost completely disappeared when ionomycin was added (Fig. 4c). Activation by transiently overexpressed SEF2-1B+ was also strongly inhibited by ionomycin (data not shown), whereas activation of luciferase expression from MEF1 sites (Fig. 4a) by transiently overexpressed MyoD was essentially unaffected by ionomycin (Fig. 4c; 80.3 $\pm$ 12.2% activation remained). Neither was activation by MyoD plus E47 nor transcription due to the constitutive overexpression of Myc in the Burkitt's lymphoma significantly affected (data not shown). Thus, the increased Ca²⁺ level selectively decreased transcriptional activation *in vivo* for bHLH proteins that are Ca²⁺/CaM sensitive *in vitro*.

Inhibition by CaM could occur either by inhibited formation of DNA-binding dimers of bHLH domains, or by blocked contacts between dimers and the nucleotide sequence. Most known CaM-binding structures are basic, amphiphilic and α -helical¹⁹. The basic DNA-binding sequence of the bHLH, which has several hydrophobic residues, and the amphiphilic helix 1, which has a basic side and an acidic side in E12/E47 and SEF2-1/ITF^{5,6,11}, are therefore both related to CaM targets. However, a peptide corresponding to the basic sequence of SEF2-1 was unable to block CaM inhibition (data not shown), indicating that CaM affects the HLH oligomerization structure. The decrease or complete block of CaM inhibition of an intrinsically CaM-sensitive bHLH in some hetero-oligomers (Fig. 3d, e), and decreased crosslinking of bHLH dimers on CaM addition (Fig. 2b), also favours involvement of oligomer interface structures. In conclusion, this study has identified a system where Ca²⁺ signals can regulate gene expression more directly than previously anticipated, through selective Ca²⁺-dependent CaM inhibition of bHLH DNA-binding domains. Signals affecting the concentration of free Ca²⁺-loaded calmodulin may thus lead to distinct regulatory responses through the differential effects on various bHLH homo-oligomers and hetero-oligomers. □

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Structure of the regulatory domains of the Src-family tyrosine kinase Lck

Michael J. Eck*†, Shane K. Atwell*, Steven E. Shoelson‡ & Stephen C. Harrison*||

* Howard Hughes Medical Institute and Laboratory of Molecular Medicine, Children's Hospital, 300 Longwood Avenue, Boston, Massachusetts 02115, USA

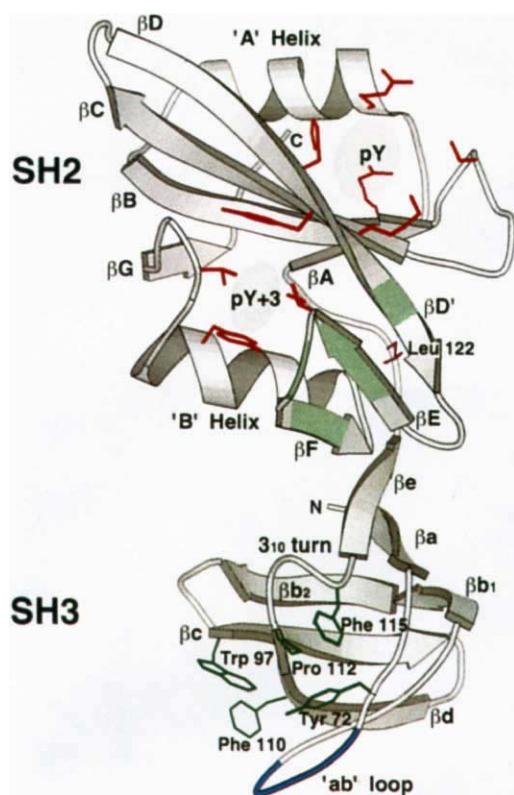
† Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

‡ Research Division, Joslin Diabetes Center, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

|| Department of Biochemistry & Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, USA

THE kinase p56^{lck} (Lck) is a T-lymphocyte-specific member of the Src family of non-receptor tyrosine kinases¹. Members of the Src family each contain unique amino-terminal regions, followed by Src-homology domains SH3 and SH2, and a tyrosine kinase domain. SH3 and SH2 domains mediate critical protein interactions in many signal-transducing pathways². They are small, independently folded modules of about 60 and 100 residues, respectively, and they are often but not always found together in the same molecule. Like all nine Src-family kinases (reviewed in ref. 3), Lck is regulated by phosphorylation of a tyrosine in the short C-terminal tail of its catalytic domain⁴. There is evidence that binding of the phosphorylated tail to the SH2 domain inhibits catalytic activity of the kinase domain^{5,6} and that the SH3 and SH2 domains may act together to effect this regulation⁷. Here we report the crystal structures for a fragment of Lck bearing its SH3 and SH2 domains, alone and in complex with a phosphotyrosyl peptide containing the sequence of the Lck C-terminal regulatory tail. The latter complex represents the regulatory apparatus of Lck. The SH3-SH2 fragment forms similar dimers in both crystals, and the tail peptide binds at the intermolecular SH3/SH2 contact. The two structures show how an SH3 domain might recognize a specific target and suggest how dimerization could play a role in regulating Src-family kinases.

The 60-residue SH3 domain is a compact antiparallel β -sandwich composed of five strands β a- β e, a helical turn, and a long β -hairpin-like loop^{8–10}. A putative ligand-binding site has been identified on the surface of SH3 domains based on locations of conserved residues¹⁰ and on shifts in NMR spectra recorded in the presence of proline-rich ligand peptides^{8,9}. The site is a hydrophobic 'pouch' on the surface of the domain. The five residues that line this pouch in the Lck SH3 are shown in green in Fig. 1 (Tyr 72, Trp 97, Phe 110, Pro 112, Phe 115).



◀ FIG. 1 Richardson diagram produced with the program MOLSCRIPT²², showing the secondary structure and relative orientations of the SH3 and SH2 domains within a single molecule. The SH2 domain is at the top of the figure; the SH3 domain below and slightly to the rear. The β -sandwich of the SH3 is seen roughly 'on edge', looking into the presumptive binding groove. Note the minimal contact between domains. On the SH2 domain, amino-acid side chains that participate in high-affinity phosphotyrosyl-peptide binding are shown in red; the phosphotyrosine (pY) and pY + 3 binding pockets are labelled. The side chain of Leu 122, the first residue in the hydrophobic core of the SH2 domain, is shown in purple. The regions of the SH2 domain that interact with the SH3 domain at a dimer contact are highlighted in green. SH3 residues that are likely to participate in ligand binding (see text) are shown in green, and the 'ab' loop, which contacts the phosphopeptide in our crystalline complex, is highlighted in blue. Lower-case letters are used to label the β -strands of the SH3 domain, and upper-case letters are used for the SH2 domain. Turns are named according to the secondary structure elements that they join. The N and C termini are labelled.

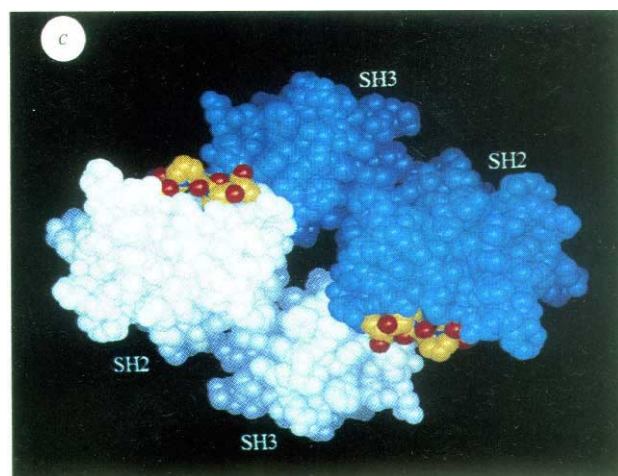
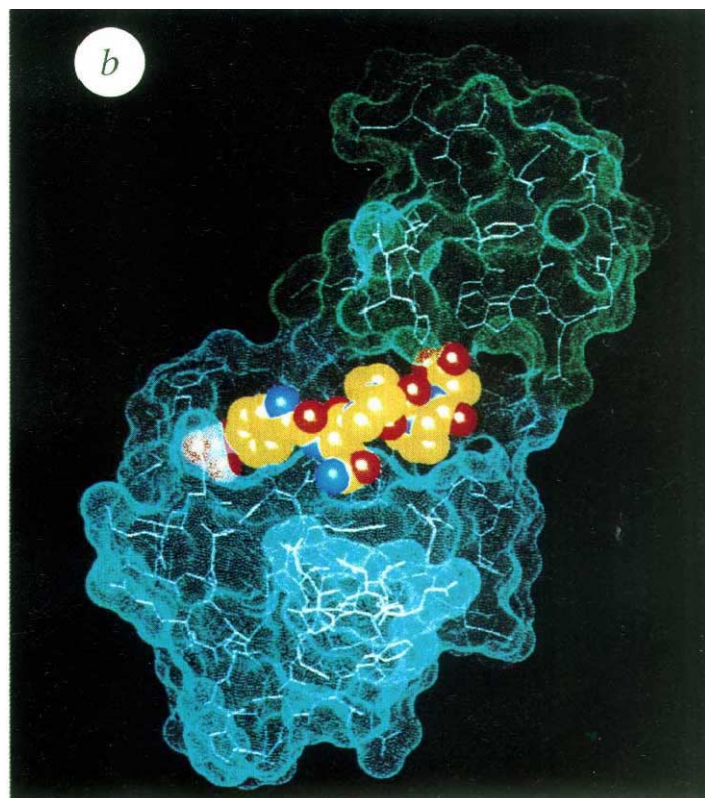
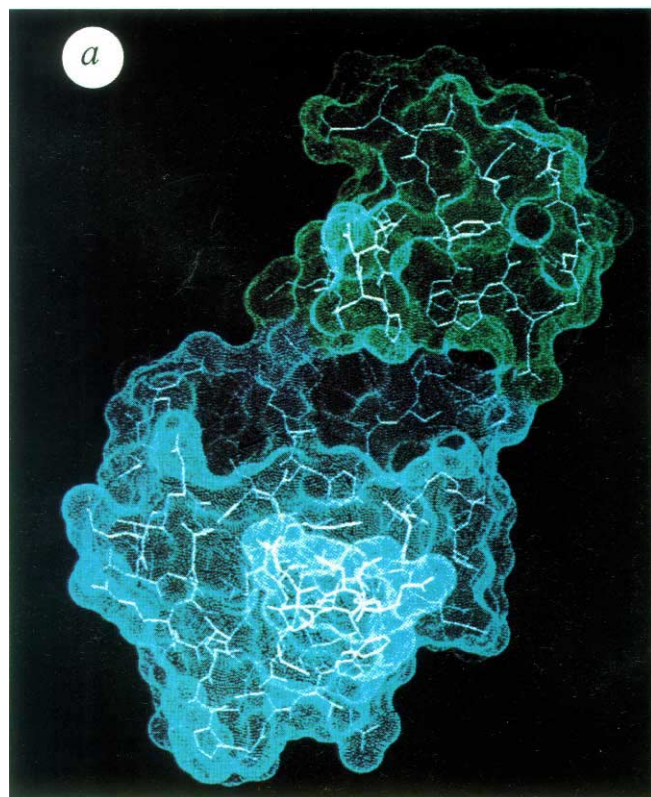


FIG. 2 a and b, Solvent-accessible surface²³ of the dimer-related SH3 and SH2 domains of the liganded structure. The SH3 surface is coloured green, the SH2 blue. The two domains together form the groove that binds the phosphorylated tail peptide. In b, a space-filling model of the peptide is added to the surface in a. c, Space-filling model of the SH3-SH2/tail structure, showing the 'head-to-tail' dimer interactions. The orientation is similar to that in a and b. Both domains of the one molecule are coloured blue, the dimer-related molecule is white. The

tail peptide is coloured by atom type. Note the extensive interaction at the dimer interface and the relatively loose interaction between covalently linked SH3 and SH2 domains. The unliganded structure reveals essentially identical head-to-tail dimeric packing interactions. (Figure prepared using Insight II, Biosym, Inc.)

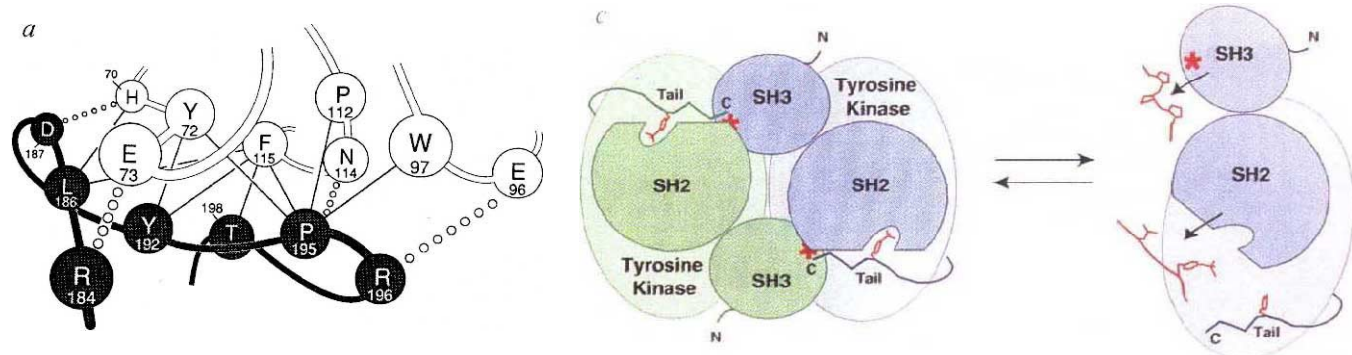
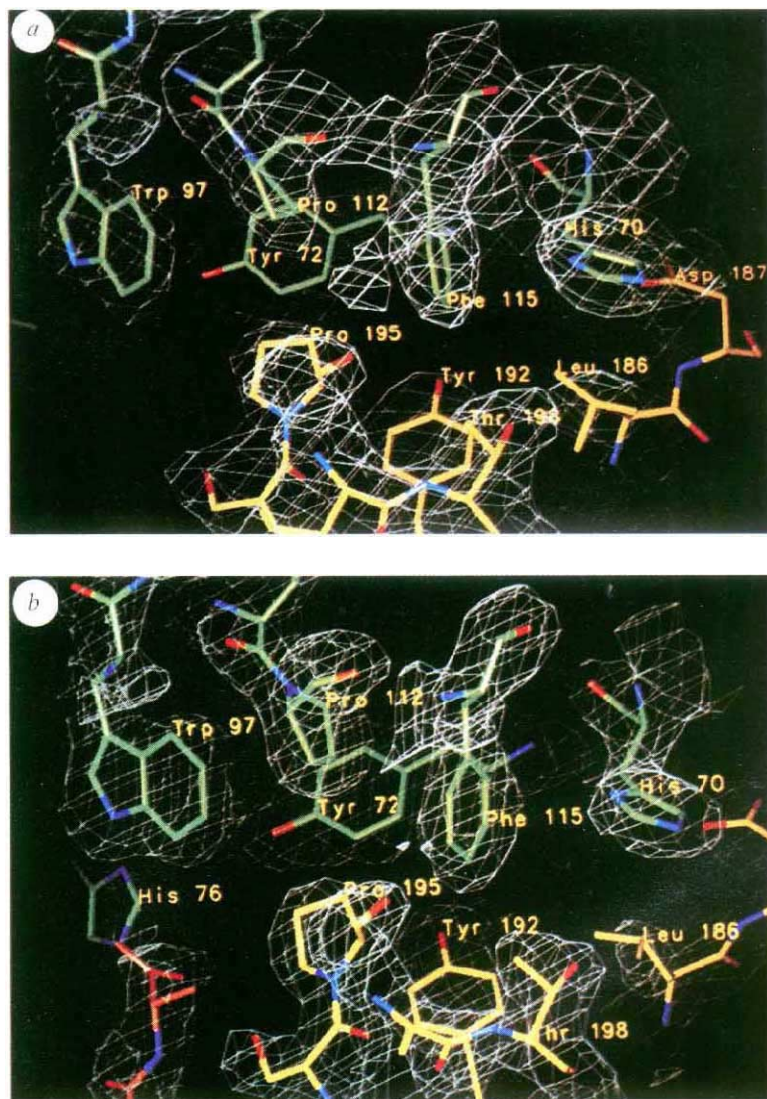


FIG. 4 Electron density maps and refined atomic models for *a*, the SH3-SH2 structure, and *b*, the SH3-SH2/tail structure. Both maps were calculated with $(2F_o - F_c)$ coefficients and phases from the refined atomic models and show a region at the interface between dimer-related SH3 and SH2 domains. The model for the SH3 domain is coloured green, the SH2 yellow, and in the liganded structure the peptide is coloured orange.

METHODS. The SH3-SH2 protein (residues 53-226) was produced in a T7-based bacterial expression system and purified by phosphotyrosine affinity essentially as described for the Lck SH2 domain¹⁴. The protein was maintained in a storage buffer containing 200 mM NaCl, 50 mM Tris, pH 8.5, 5 mM dithiothreitol, and 0.02% sodium azide. Unliganded crystals (space group $P3_121$; $a = 82.51$ Å, $c = 116.22$ Å) were grown in hanging drops by combining 1 μ l of protein solution (15 mg ml⁻¹ SH3-SH2 protein in storage buffer plus 10 mM *o*-phospho-L-tyrosine) with 1 μ l reservoir solution containing 1.1 M sodium tartrate, 100 mM Tris, pH 8.5, and 0.01% 2-mercaptoethanol. Liganded crystals (space group $R32$, $a = 72.35$ Å, $c = 187.36$ Å) were grown in hanging drops by combining 2 μ l protein/peptide solution (13 mg ml⁻¹ SH3-SH2 protein in storage buffer plus 6 mM phosphopeptide TEGQpYQPQPA) with 2 μ l of a well solution containing 950 mM sodium tartrate, 400 mM ammonium sulphate, and 100 mM Tris, pH 8.5. The phosphotyrosyl peptide was prepared as described²⁵. Data collection: Diffraction data for both structures were recorded at room temperature on a Mar Research image plate scanner mounted on an Elliot GX-13 rotating anode generator. Each data set was collected from a single crystal. One degree oscillation images were indexed, integrated and scaled using the program XDS²⁶. Both the liganded and unliganded structures were determined using molecular replacement. SH3-SH2 structure determination: The Lck SH2 domain¹⁴, with phosphopeptide and solvent molecules omitted, was used as an initial search model. Rotation functions and translation searches were calculated with X-PLOR²⁷. With the two SH2 domains oriented and positioned in the asymmetric unit, we used the spectrin SH3 domain¹⁰ as a search model in Patterson rotation and translation functions calculated with AMoRe²⁸. About 450 rotation peaks culled from searches calculated using a range of vector lengths were tested in translation searches, and a single rotation/translation solution had a correlation coefficient obviously above background. This solution placed the SH3 domain plausibly in the lattice, and a second SH3 domain was positioned using the non-crystallographic symmetry operator (calculated from the SH2 domains). Rigid body refinement with all four domains yielded an *R*-factor of 42.6% and a correlation coefficient of 0.515. The initial molecular replacement phases and electron density were improved by iterative real-space non-crystallographic symmetry averaging using the program RAVE²⁹, and the model was refined with multiple cycles of manual re-fitting and simulated annealing and positional refinement using X-PLOR²⁷. The program O (ref. 30) was used for all model building. Annealed omit maps were used to refit several regions of the model, and tight non-crystallographic symmetry restraints were maintained during simulated annealing refinement steps. Restrained, individual temperature factors were refined. The current model has excellent geometry and contains residues 63-226 of Lck for both molecules in the asymmetric unit and no solvent molecules. The crystallographic *R* factor is 19.6% ($R_{free} = 32.4\%$) for all data between 6.0 and



3.0 Å resolution. Although the crystallization required phosphotyrosine, no electron density was visible in the phosphotyrosine-binding pocket of the SH2 domain. SH3-SH2/tail structure determination: The SH3-SH2 structure was used as a search model in molecular replacement calculations carried out using X-PLOR²⁷. Rigid-body minimization of the properly rotated and translated model yielded a crystallographic *R* value of 42.2% for data between 12.0 and 3.2 Å resolution. The phosphopeptide model was built into the electron density map after an initial cycle of simulated annealing refinement. The model was further refined with multiple cycles of manual re-fitting³⁰ and simulated annealing and positional refinement²⁷. Restrained individual temperature factors were refined. The current model has excellent geometry and contains residues 63-226 of Lck, residues pY to pY + 5 of the phosphopeptide, and 58 water molecules. The crystallographic *R* factor is 19.0% ($R_{free} = 32.9\%$) for all data between 5.0 and 2.5 Å resolution.

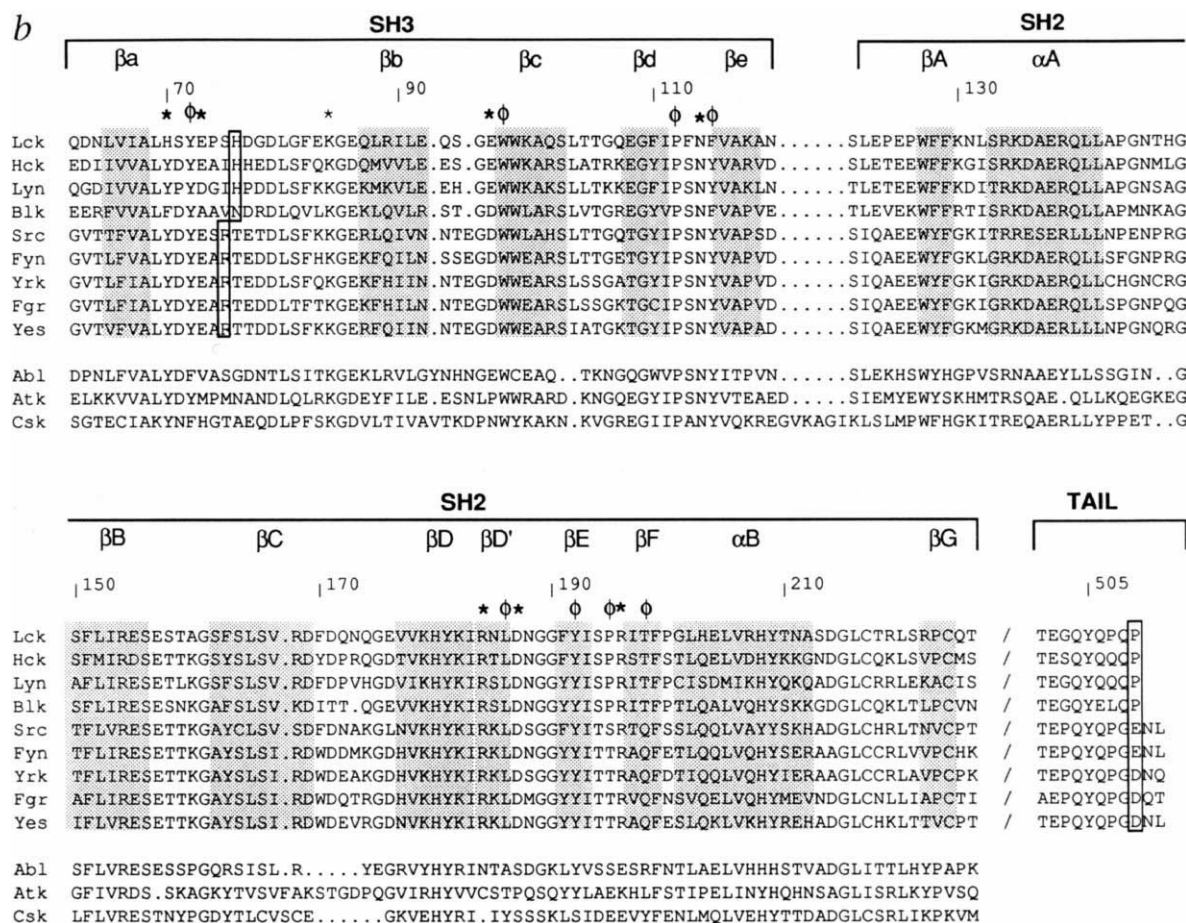


FIG. 3 a, Diagram showing non-covalent interactions at the SH3/SH2 dimer interface. Open circles show residues in the SH3 domain; filled circles the SH2 domain. Solid lines indicate non-polar contacts across the interface; dotted lines indicate hydrogen bonds or salt bridges. Each SH3/SH2 dimer contact buries $\sim 500 \text{ \AA}^2$ on each domain, or a total of $\sim 2,000 \text{ \AA}^2$ (refs 23, 24). This is comparable to the buried surface in relatively strong protein/protein interactions²⁴. By contrast, the interface between covalently linked SH3 and SH2 domains in our structure buries only $\sim 225 \text{ \AA}^2$ per domain. b, Aligned sequences of the SH3, SH2 and tail regions of the nine Src-family tyrosine kinases, and of Abl, Atk and Csk. The last three kinases have consecutive SH3 and SH2 domains, but unlike the Src-family members lack a regulatory C-terminal tail. Regions of β -sheet and α -helix are shaded and labelled within each domain. Residue numbering is indicated for Lck. Residues that participate in hydrophobic and polar interactions at the dimer interface are indicated by a ϕ and an asterisk, respectively. Polar interactions stabilizing the interface include salt bridges (Glu 73–Arg 184, Arg 196–Glu 96, Asp 187–His 70 or Lys 84) and a hydrogen bond (Asn 114–Pro 195 carbonyl). Note that these interactions are conserved within the Src family, but not in more distantly related kinases that lack regulatory tails. Residues in the 'ab' loop of the SH3 domain and the C-terminal tail that are likely to form an ion-pair are boxed (see text). Histidine 76 in the 'ab' loop forms a salt bridge with the terminal carboxyl group of the peptide in our structure. This peptide is one residue longer than the actual Lck tail, but the histidine side chain could equally well interact with the terminal carboxyl of the authentic peptide. The length of the tail is the same in other members of the Lck subfamily, and the histidine is present in all but Blk (where an Arg two residues later could substitute). In the Src subfamily, the tail is two residues longer, but a negatively charged residue is present at the position corresponding to the C terminus of Lck, and an arginine in the SH3 domain (at the position of Ser 75 in Lck) could salt-bridge to it. This arginine is

the site of mutations that affect Src regulation (see text). c, Diagram showing how dimerization could regulate Src-family kinases. The SH3, SH2 and catalytic domains are shown schematically. The tail peptides are shown with a tyrosine side chain. A red star in the SH3 domain marks the location of its ligand binding surface and of the adjacent 'ab' loop, which contacts the tail peptide in our crystals. The N-terminal domains, which are myristoylated and bound to the cytoplasmic tails of CD4 or CD8, are not shown. The view of the dimer is from the membrane. The N termini of the SH3 domains turn out of the plane of the page (towards the membrane) and therefore could connect to the membrane-associated N-terminal domains. The structure suggests that phosphorylation of Tyr 505 could stabilize a dimer through SH3/tail interactions. The tail peptide interacts with both the SH2 and SH3 domains, increasing the total buried surface area of the interaction. In addition to tethering and perhaps inhibiting the kinase domain, the closed structure would leave the SH2 domain occupied and unavailable to bind a heterologous target, and the SH3 domain sequestered with its ligand-binding surface buried by the SH2 domain and unable to recognize a target proline-rich ligand. A number of events might trigger a transition to an 'open' state: dephosphorylation of Tyr 505, which would unblock the SH2 domain and probably weaken the dimer contact; apposition of a 'high-affinity' SH2 ligand, which could compete for binding with the tail peptide and repel the SH3 domain; or apposition of a ligand for the SH3 domain, which might dissociate the closed structure independently of any phosphorylation or dephosphorylation event. In an open state, the SH3 and SH2 domains would be free to bind their respective ligands. Autophosphorylation of the catalytic domain (Tyr 394 in Lck) is also important for full activation of kinase activity³. The SH3/SH2 dimer interactions seen here might orient the catalytic domains in a manner that would prevent cross-autophosphorylation. We note that an inactive monomeric state can readily exist, if the C-terminal tail binds to the SH2 domain of the same polypeptide.

The 100-residue SH2 domain contains a central β -pleated sheet flanked by α -helices A and B (refs 11–14). The proximal face of the central sheet, the BC loop, and the amino-terminal residues of the A helix form a phosphotyrosine binding pocket. The distal face of the central sheet and the opposing 'jaws' made by the EF and BG loops create a second pocket capable of accepting a hydrophobic side chain (Fig. 1). The SH2 domains of Lck and Src bind particularly strongly to peptides containing the sequence pYEEI (ref. 15), and the structures of these domains with a bound 'YEEI' phosphopeptide show the peptide in an extended conformation and the phosphotyrosine (pY) and isoleucine (pY + 3) inserted into the pockets just described^{14,16}.

The polypeptide segment linking the two domains is quite short. Only two amino acids intervene between the last residue of strand β e in the SH3 domain and Leu 122, which forms part of the SH2 hydrophobic core. The corresponding residue is either a leucine or isoleucine in all nine Src family members, which also have identical spacings between their SH3 and SH2 domains.

The consecutive SH3 and SH2 domains make very little contact with each other, aside from the polypeptide chain linking them (Fig. 1). This limited contact includes a hydrogen bond from the main-chain amide of Lys 118 in the SH3 domain to the side chain of Glu 204 in the SH2 domain, and in the liganded molecule a hydrogen bond from Arg 207 to the carbonyl of Val 116. In addition, Pro 200 in the SH2 domain is in contact with the side chains of Leu 69 and Ala 117 in the SH3 domain. The α -carbons of Tyr 181 (β D5) and Pro 112 (de1), which mark the centres of the ligand-binding regions of the SH2 and SH3 domains, respectively, are 31 Å apart. The two domains could in principle bind to sites on the surface of a single larger ligand molecule or to successive proline and phosphotyrosine motifs on an extended peptide loop. But given the paucity of interactions between the two domains, we expect the link between them to be flexible, allowing interaction of the two domains with separate ligands.

The unit cell contains two crystallographically independent SH3–SH2 molecules. These two molecules have essentially identical conformations, and they pack together in the crystal related by a non-crystallographic 2-fold symmetry axis. This arrangement creates a 'head-to-tail' interaction between the two molecules, in which the SH3 of one molecule binds the SH2 of the other and vice versa (Fig. 2). In contrast to the minimal contact region seen between covalently linked SH3 and SH2 domains, the intermolecular SH3/SH2 contacts in the dimer are extensive, creating a large solvent-excluded area. The contact contains a large central hydrophobic core surrounded by a number of hydrogen bonds and salt bridges (Fig. 3a). On the SH3 domain, the interface includes residues from the 'ab' loop, the helical 'de' turn, and the 'bc' loop. On the SH2 domain, it includes portions of the β D', β E and β F strands and the EF turn (one of the 'jaws' of the pY + 3 pocket).

The structure with the bound 'tail' peptide has the same overall arrangement of domains as in the unliganded SH3–SH2 fragment. There is only one molecule in the R32 asymmetric unit, but the crystallographic 2-fold axis generates a dimer that is nearly identical to the non-crystallographic dimer. Other crystal lattice contacts are very different. The contact between dimer-related SH3 and SH2 domains is as well conserved as the one between covalently linked domains. The phosphotyrosyl peptide (sequence TEGQpYQPQPA) binds at the intermolecular SH3/SH2 contact (Fig. 2), and interacts with both domains. The phosphotyrosine side chain inserts into the pY pocket of the SH2 domain exactly as it does in the SH2/peptide complexes, and most of the interactions previously described are present. Arginine α A2, which in the high-affinity YEEI complex participates in a number of hydrogen bonds, including an amino-aromatic bond to the phosphotyrosine ring¹³, here donates only one hydrogen bond to the phosphate from its N ϵ ; the guanidinium group turns away from the ligand. The conformation of the

rest of the peptide differs substantially from that of the YEEI peptide bound to the Lck SH2 (ref. 14). Instead of forming an extended chain across the D-strand with anchors at pY and pY + 3, the peptide appears to follow the seam between the SH2 domain and the apposed SH3 domain, with contacts to both domains along its length. The first four residues of the peptide are poorly ordered and are not included in the current model. The glutamine at pY + 1 adopts a conformation very similar to that of the glutamic acid at the corresponding position in the YEEI peptide complexes of Lck and Src, but the backbone of the peptide between pY + 1 and pY + 3 and is kinked by Pro pY + 2, and it is not in close contact with the surface of the SH2 domain. The side chain of Pro pY + 4 packs into the top of the pY + 3 binding pocket. Histidine 76 (ab8) on the SH3 domain is positioned to bind the C-terminal carboxylate of the peptide.

The dimer interface between the two domains (Figs 3 and 4) suggests how an SH3 domain might recognize a proline-rich ligand. Proline 195 (EF2) on the SH2 domain projects into the hydrophobic pouch of the apposing SH3 domain. The proline lies in a van der Waals pocket defined by the side chains of Try 72, Trp 97, Pro 112, and Phe 115. Additional hydrophobic contacts with the SH3 are made by SH2 residues Leu 186, Tyr 192, and Thr 198. It is possible that similar interactions might be made by the proline-rich sites believed to be the targets for SH3 domains¹⁷. As a motif for protein–protein interactions, proline-rich peptides might be rationalized as being a source of soluble hydrophobic residues. Interspersed non-prolyl residues might account for specificity. One target for the Lck SH3 domain is phosphatidylinositol-3-OH kinase¹⁸: two sequences within the p85 chain of this kinase (PPTPKRPPRPLP and APALPPKPPKP) are known to bind the Fyn SH3 domain and have been proposed as the likely binding site for the Lck SH3 as well¹⁸. Both contain basic residues interspersed with proline residues. Model building suggests that the PRPPRP sequence could make salt-bridge and van der Waals interactions with the Lck SH3 domain that are similar to those made by Arg 184, Tyr 192, Pro 195 and Arg 196 of the apposed Lck SH2 domain.

Does the dimeric association of SH3–SH2 fragments reveal an interaction that actually occurs at some stage of activation or inactivation in the Lck signal transduction pathway? We have not detected dimerization in solution of the SH3–SH2 protein in the presence or absence of phosphopeptide, and Src-family kinases are monomeric in detergent lysates¹⁹. But dimer formation will be favoured in the intact cell because the Src-family kinases are tethered to the membrane. Moreover, the Fyn SH3 domain binds directly to bead-immobilized Fyn SH2 or SH3–SH2 (H. Band, personal communication). In addition, the extent and intimacy of the intermolecular SH3/SH2 contact and its conservation in two otherwise different crystal forms suggest that it might be more than an adventitious packing interaction: (1) it is significantly more complementary than typical lattice interactions (Figs 2, 3 and 4); (2) most of the residues at the interface, including those involved in salt bridges and hydrogen bonds, are conserved within the Src family, but diverge in other molecules that contain adjacent SH3–SH2 domains, such as kinases with no regulatory tail (Fig. 3); (3) SH3 residues that contact the tail peptide are conserved in the Lck subfamily (which has one type of tail sequence), and they are characteristically different in the Src subfamily (which has a different type of tail sequence) (Fig. 3b); (4) the dimeric interaction of the ab loop in the SH3 domain with the tail peptide provides a structural explanation for the transforming potential of mutations in the corresponding loop in the Src SH3 domain^{20,21}. Introduction of even single amino-acid changes in the SH3 ab loop is sufficient to activate the catalytic activity of c-Src^{7,21}. Moreover, one of these mutations, Arg 95 to Trp, diminishes the ability of Src to be regulated by phosphorylation of its C-terminal tail (Y527) (ref. 7). Our dimeric structure suggests that this mutation would destroy a salt bridge between Arg 95 and pY + 4 in the tail and that it might also sterically hinder interaction of the SH3 with

the SH2 domain. In contrast, within a monomeric SH3-SH2/tail complex, the ab loop on the SH3 domain is remote from the phosphopeptide (Fig. 1), and any contact between the two seems unlikely, given the short hinge region between the covalently adjacent domains. In particular, the intermolecular SH3/S2 contact in our crystals could not form intramolecularly without substantial unfolding of one or both domains.

These conservations and correlations lead us to propose that the dimer structure may represent a model for the regulatory regions in a 'closed' state of Src-family kinases. In this state, the SH3 and SH2 domains and the C-terminal tails would all be sequestered and the kinase domains presumably oriented to prevent cross phosphorylation (Fig. 3c). Transition to an open, probably monomeric, state would free the SH3 and SH2 domains to bind heterologous ligands and to direct, together with the unique N-terminal segment, the specificity and cellular localization of the active kinase. □

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Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II

Ronny Drapkin*, Joyce T. Reardon†, Athar Ansari*, Juch-Chin Huang†, Leigh Zawel*, KyuJeong Ahn†, Aziz Sancar† & Danny Reinberg*†

* Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, 675 Hoes Lane, Piscataway, New Jersey 08854-5635, USA

† Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, USA

THE RNA polymerase II general transcription factor TFIIH is composed of several polypeptides. The observation that the largest subunit of TFIIH is the excision-repair protein XPB/ERCC3 (ref. 1), a helicase implicated in the human DNA-repair disorders xeroderma pigmentosum (XP) and Cockayne's syndrome^{2,3}, suggests a functional link between transcription and DNA repair^{4,5}. To understand the connection between these two cellular processes, we have extensively purified and functionally analysed TFIIH. We find that TFIIH has a dual role, being required for basal transcription of class II genes and for participation in DNA-excision repair. TFIIH is shown to complement three different cell extracts deficient in excision repair: XPB/ERCC3, XPC and XPD/ERCC2. The complementation of XPB and XPD is a consequence of ERCC3 and ERCC2 being integral subunits of TFIIH, whereas complementation of XPC is due to an association of this polypeptide with TFIIH. We found that the general transcription factor TFIIE negatively modulates the helicase activity of TFIIH through a direct interaction between TFIIE and the ERCC3 subunit of TFIIH.

TFIIH was purified from HeLa cell nuclear extract as shown in Fig. 1a. Silver staining of the transcriptionally active pool from the final purification step revealed the presence of several

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polypeptides (Fig. 1d, lane 1). Of these polypeptides, three could be correlated with proteins already described. As found previously¹, the 89K polypeptide was shown to be ERCC3 by western analysis (Fig. 1d, lane 2). The 62K subunit of TFIIH was detected by western blotting and silver staining⁶ (Fig. 1d, lanes 1 and 2), as expected. Interestingly, antibodies directed against ERCC2 identified a polypeptide with an R_f value of ~80K (Fig. 1d, lane 2) which was not detectable by silver staining. Thus, the polypeptide composition of TFIIH appears to contain, in addition to p62, two polypeptides known to participate in excision repair, ERCC2 and ERCC3. We also identified a polypeptide of M_r 56K in the TFIIH preparation (Fig. 1d, asterisk). This polypeptide is the largest subunit of TFIIE⁷⁻⁹ as determined by western analysis (data not shown, and see below).

To analyse the polypeptide composition of TFIIH further, we did the following experiments. First, we investigated whether ERCC2, ERCC3 and p62 coeluted with TFIIH activity during the different steps of purification; Fig. 1a shows the coelution on phenyl-Superose chromatography of these polypeptides with TFIIH activity. Second, we tested whether ERCC2 and/or ERCC3 could exist in a free form not associated with the TFIIH complex using quantitative western analysis; we found all ERCC3-reacting material in nuclear extract copurifying with TFIIH activity. The result was similar with ERCC2, but ~5% of ERCC2 was detected as free polypeptide upon fractionation on a gel filtration column (data not shown). Third, antibodies against ERCC2 not only immunoprecipitated ERCC2, but also ERCC3 and the 62K subunits of TFIIH (Fig. 1b). Finally, the ratio of ERCC2 and ERCC3 to p62 during the different steps of purification was found to be constant in the TFIIH fractions (Fig. 1c).

Having established that ERCC2 and ERCC3 are components of TFIIH, we investigated the possible role of ERCC2 and ERCC3 in transcription. Addition of antibodies against ERCC2 or ERCC3 to the reconstituted transcription system resulted in an inhibition of transcription (Fig. 2). This inhibition was specific because it could be overcome by addition of excess TFIIH, but not by TFIIB or TFIIE (Fig. 2). These results are consistent with the observation that the yeast homologues of ERCC2 (RAD3) and ERCC3 (RAD25) are essential for transcription^{10,11}.

Our finding that TFIIH contains at least two excision-repair proteins suggested that the entire TFIIH complex may partici-

† To whom correspondence should be addressed.

pate in excision repair. The human excinuclease removes DNA damage by hydrolysing the fifth phosphodiester bond 3' and the 21–23rd phosphodiester bond 5' to the lesion¹². These incisions release a damaged DNA fragment of 27–29 nucleotides. Using an assay that measures the release of such a fragment, extracts derived from all seven XP complementation groups (XPA–XPG) (reviewed in refs 13–15) including the rodent ERCC1 mutant, were tested for excision-repair complementation using

the TFIIH fraction from the last chromatographic step. Only XPB, XPC and XPD cell-free extracts were complemented by TFIIH (Fig. 3a). Importantly, there was an absolute coelution of TFIIH transcription activity with activities complementing XPB and XPD extracts in the last purification step (Fig. 3b, c and e), whereas the XPC complementing activity is shifted by three fractions (Fig. 3d). We conclude that XPB (ERCC3) and XPD (ERCC2) are integral subunits of TFIIH and that XPC,

FIG. 1 a, TFIIH transcription activity copurifies with ERCC3, ERCC2 and p62 polypeptides. TFIIH was purified as described²⁶ with modifications. Transcription reactions contained the adenovirus major late promoter (Ad-MLP) directing transcription of a 392-nucleotide G-less cassette. Reactions contained all the purified general transcription factors (GTFs) and RNA polymerase II, except TFIIH. Assays were supplemented with the fraction whose number is indicated at the top of each lane. 'I', input to the column; 'FT', flow-through of the column. Co-elution of ERCC2, ERCC3 and p62 polypeptides was confirmed by western analysis. The p62 panel represents a western done with protein-A-purified p62 antibodies; ERCC2 and ERCC3 panels are westerns using affinity-purified antibodies against ERCC2 and ERCC3. **b**, Affinity-purified ERCC2 antibodies immunoprecipitate the TFIIH subunits p62 and ERCC3. NS, nonspecific band present in lane 1 and in lane 2. **c**, Quantitative western blot of p62, ERCC2 and ERCC3 polypeptides during purification. Equal amounts of p62-reactive material, as determined by quantification on a Biorad GS-250 Molecular Imager, were loaded in each lane and western blotted using all three antibodies (against p62, ERCC2, ERCC3). **d**, Composition of TFIIH. The transcriptionally active pool from the final chromatography step was subjected to SDS-PAGE followed by silver staining (lane 1). The same pool was analysed by western blotting using all three antibodies; the resulting blot is shown next to the silver-stained lane (lane 2). Size markers are shown in lane 3.

METHODS. **a**, TFIIH was purified from 10.5 g HeLa nuclear extract and fractionated on phosphocellulose as described^{26,27}. **b**, 50 μ l TFIIH eluted from the Superdex-200 column was incubated for 3 h at 4 °C with either protein A-agarose beads (RepliGen) alone (lane 2) or with protein A-agarose beads to which antibodies against ERCC2 had been adsorbed (lane 1). The beads were then washed 5 times with buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM NaF, 100 μ M NaVO₃, 10 mM β -glycerophosphate, 1.0% NP-40, 0.3 M NaCl, and resuspended in SDS-PAGE loading buffer. After electrophoresis proteins were transferred onto nitrocellulose and the ERCC3, ERCC2 and p62 components of TFIIH were detected with the appropriate antibodies. Lane 1 contains 10 μ l of the TFIIH phenyl-Superose (last step of purification) fraction as a positive control. **c**, Quantitative western blot. Equal amounts of p62-reactive material, independently determined, were loaded in each lane: SP-Sepharose (10 μ g), phenyl-Superose (8.5 μ g), Superdex 200 (7.7 μ g)

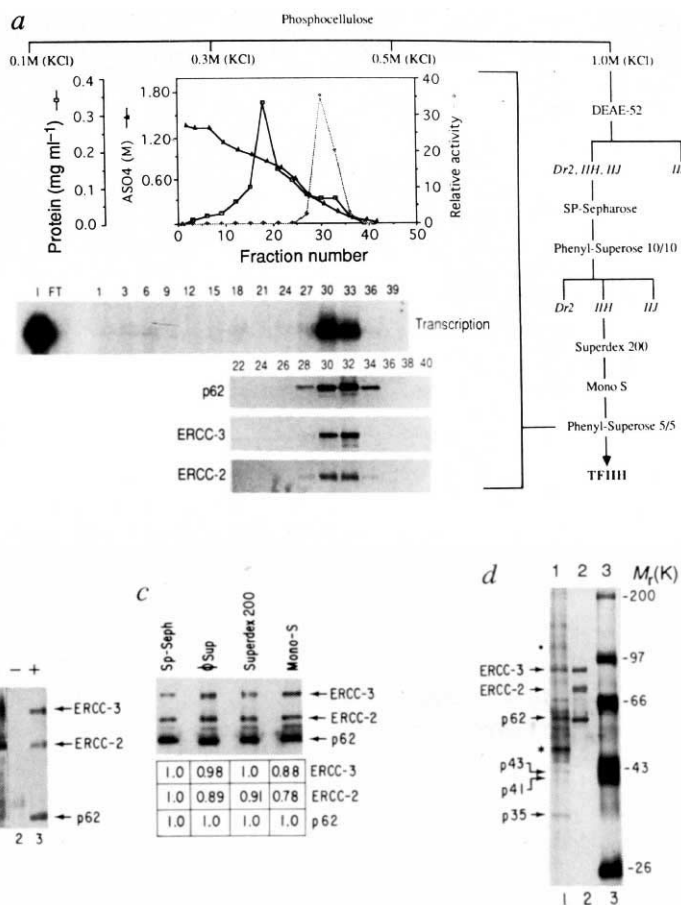
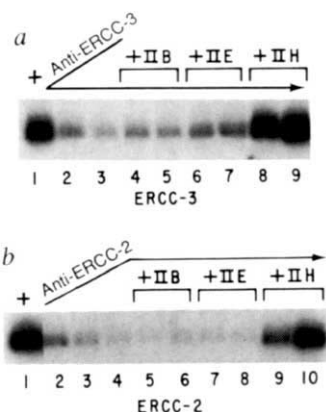


FIG. 2 Antibodies directed against ERCC2 and ERCC3 inhibit transcription. **a**, Transcription reactions were reconstituted using the general transcription factors (GTFs) and the Ad-MLP. Lane 1 is a positive control for TFIIH-dependent transcription. Lanes 2 and 3, 220 ng and 440 ng affinity-purified ERCC3 antibodies. Other lanes contain 220 ng ERCC3 antibodies in the presence of excess factors: 4 and 5, 2- and 4-fold excess TFIIH, respectively; 6 and 7, 2- and 4-fold excess TFIIIE; 8 and 9, 1- and 2-fold excess purified TFIIH (1-fold is the amount of factor saturating the assay). The excess factor was not incubated with the antibodies. **b**, Lanes 2–4 contain 300, 600 and 900 ng affinity-purified ERCC2 antibodies, respectively. Other lanes contain 900 ng ERCC2 antibody in the presence of excess factors: 5–6, 2- and 4-fold excess TFIIH, respectively; 7 and 8, 2- and 4-fold excess TFIIIE; 9 and 10, 1- and 2-fold excess purified TFIIH.

METHODS. Each lane contained 500 ng TFIIH from the Superdex-200 chromatography step preincubated with either ERCC2 or ERCC3 antibodies for 30 min on ice. After pre-incubation, the remaining GTFs, RNA polymerase II, DNA, nucleotide mix, and excess factors were added. Transcription reactions were incubated for an additional hour at 28 °C and processed as described²⁶.

and Mono-S (6.6 μ g). Proteins were subsequently transferred onto nitrocellulose and western blotted with antibodies against p62, ERCC2 or ERCC3 using the enhanced chemical luminescence (ECL) method (Amersham). The blot was exposed to a Biorad CH screen and then imaged on a Biorad GS-250 Molecular Imager. The p62-reactive material quantified for all four column pools was equal and set at unity.



although it is tightly associated with TFIIF, is not an essential subunit because it trails behind the transcription activity in the last purification step. This finding is supported by the facts that null mutants of XPB and XPD do not exist² and the yeast mutants of XPB (RAD25) and XPD (RAD3) are conditionally lethal¹⁶. In contrast, XPC null mutants have been identified in humans¹⁷ and deletion of the XPC homologue RAD4 (ref. 18) in yeast is not lethal¹⁶, suggesting that it is not essential for transcription. Because XPC cell-free extract is complemented by TFIIF, we tested for XPC in our TFIIF preparation. Western analysis with antibodies directed against XPC confirmed the presence of the 125K polypeptide (Fig. 1d, lane 1; indicated by a dot) in our TFIIF and revealed that the amount of XPC-reactive material decreased in the last step of purification as a result of its partial separation from TFIIF (data not shown).

The observation that TFIIF contains a helicase activity¹ is not surprising as XPD/ERCC2 has a 5'-3' helicase¹⁹ and XPB/ERCC3 has a 3'-5' helicase (data not shown). We wished to determine the role of this activity in transcription and whether it is subject to regulation by other general transcription factors. As TFIIF contains a kinase specific for the carboxy terminal domain (CTD) of RNA polymerase II which is stimulated by TFIIE²⁰, we investigated the effect of this factor on TFIIF-associated helicase activity and found that it is inhibited by TFIIE (Fig. 4a and b). This inhibition seems to be selective because TFIIE can inhibit helicase activity associated with recombinant ERCC3 but not that of ERCC2 or UvrD, a factor involved in excision repair in *Escherichia coli*²¹ (Fig. 4a, b). To investigate the specificity of this inhibition further, we studied the effect of antibodies directed against the two subunits of TFIIE, p34 and p56 (refs 7-9, 22). We found that preincubation of TFIIE with antibodies against p56 effectively neutralized the

repressing effect on TFIIF helicase activity; antibodies against p34 had no effect (Fig. 4c).

Our results indicate that there could be a direct physical interaction between TFIIF (ERCC3) and TFIIE (p56). To test this possibility, TFIIE, with its 56K subunit fused to glutathione-S-transferase (GST), was incubated with TFIIF or with recombinant ERCC3 or ERCC2. We used a GST-pulldown assay (Fig. 4d legend) to demonstrate an interaction between TFIIE and ERCC3 and, as expected, with TFIIF. This interaction was specific, as recombinant ERCC2 failed to interact with TFIIE (Fig. 4d). Therefore, TFIIE can repress the helicase activity of TFIIF, an effect that is mediated through direct interaction of ERCC3 with the largest subunit of TFIIE. As TFIIE has no effect on ERCC2 helicase activity but inhibits that associated with TFIIF, we suggest that the ERCC2 helicase activity is dormant in the TFIIF complex.

The role of the TFIIF helicase in transcription is unclear, but our analysis suggests that TFIIF may act in open complex formation and/or promoter clearance by melting the DNA duplex at the promoter region. Our studies on the composition of the ternary complex have failed to identify XPB (ERCC3) or XPD (ERCC2) in the complex, ruling out a role for TFIIF during elongation (P. Kumar and D.R., unpublished results). It is possible that the CTD kinase and helicase activities of TFIIF function in a concerted fashion. Phosphorylation of CTD causes a conformational change in the preinitiation complex²³, which could result in TFIIF-TFIIE dissociation and unbarring of the helicase activity of ERCC3 (TFIIF); this disinhibition would enable TFIIF to catalyse open complex and/or promoter clearance.

The XPB (ERCC3) and XPD (ERCC2) proteins and their yeast counterparts RAD25 and RAD3, respectively, were

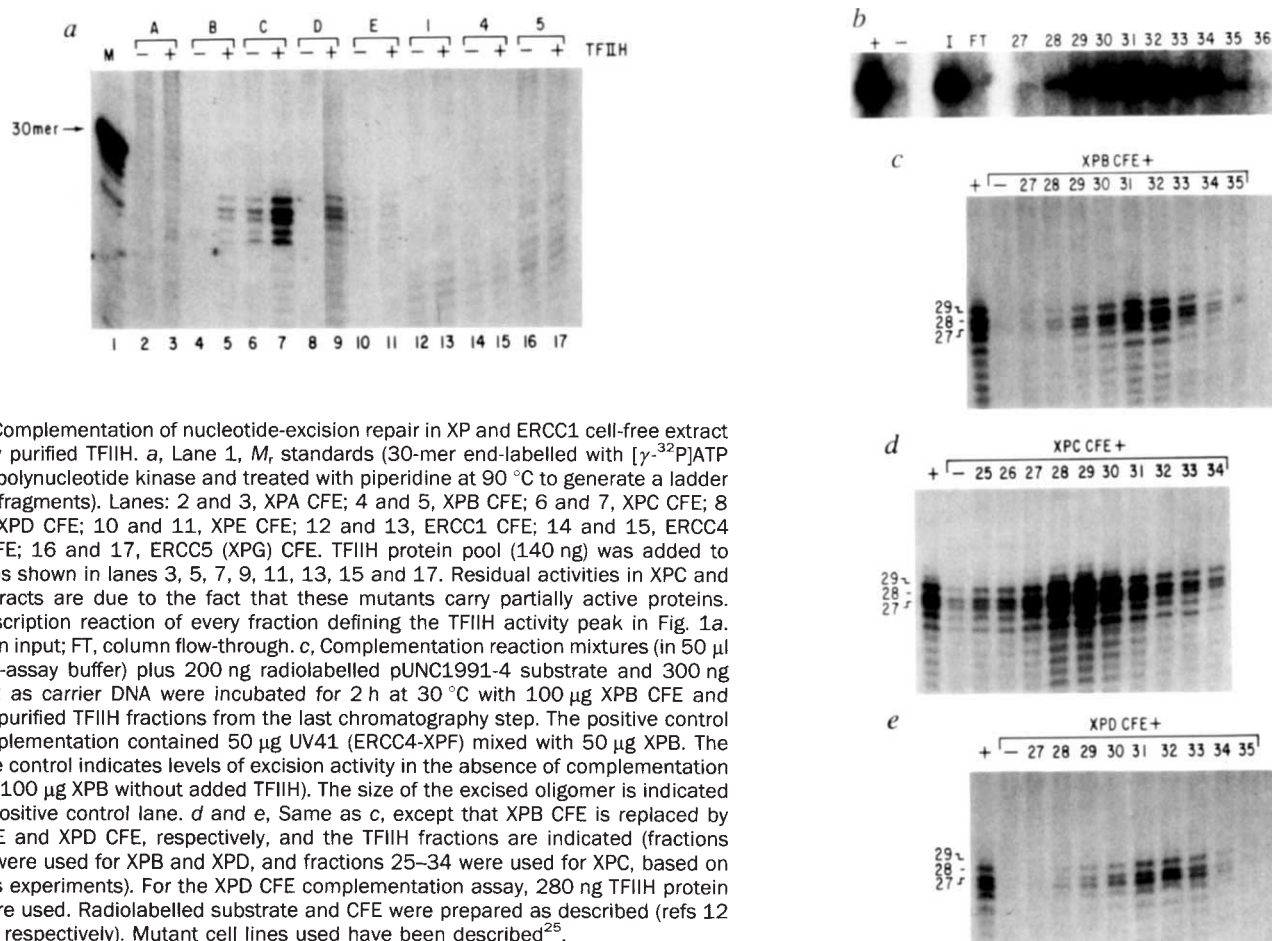
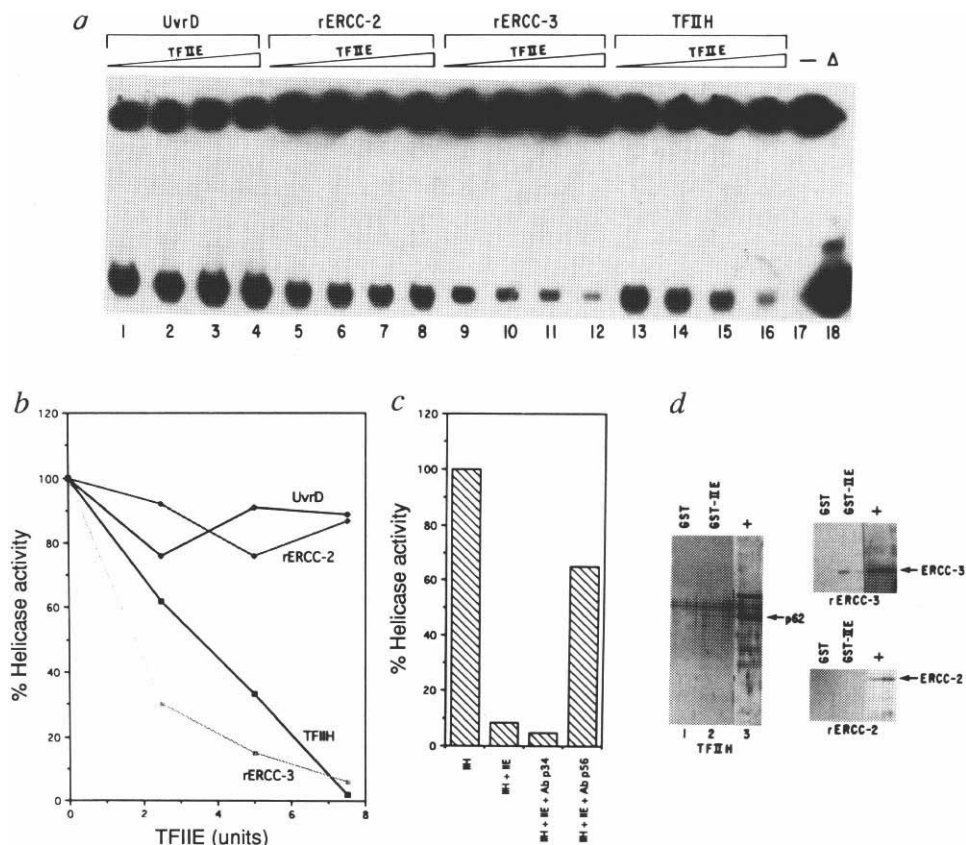


FIG. 3 Complementation of nucleotide-excision repair in XP and ERCC1 cell-free extract (CFE) by purified TFIIF. a, Lane 1, M, standards (30-mer end-labelled with [γ -³²P]ATP and T4 polynucleotide kinase and treated with piperidine at 90 °C to generate a ladder of DNA fragments). Lanes: 2 and 3, XPA CFE; 4 and 5, XPB CFE; 6 and 7, XPC CFE; 8 and 9, XPD CFE; 10 and 11, XPE CFE; 12 and 13, ERCC1 CFE; 14 and 15, ERCC4 (XPF) CFE; 16 and 17, ERCC5 (XPG) CFE. TFIIF protein pool (140 ng) was added to reactions shown in lanes 3, 5, 7, 9, 11, 13, 15 and 17. Residual activities in XPC and XPE extracts are due to the fact that these mutants carry partially active proteins. b, Transcription reaction of every fraction defining the TFIIF activity peak in Fig. 1a. I, column input; FT, column flow-through. c, Complementation reaction mixtures (in 50 μ l excision-assay buffer) plus 200 ng radiolabelled pUNC1991-4 substrate and 300 ng pBR322 as carrier DNA were incubated for 2 h at 30 °C with 100 μ g XPB CFE and 140 ng purified TFIIF fractions from the last chromatography step. The positive control for complementation contained 50 μ g UV41 (ERCC4-XPF) mixed with 50 μ g XPB. The negative control indicates levels of excision activity in the absence of complementation (that is, 100 μ g XPB without added TFIIF). The size of the excised oligomer is indicated in the positive control lane. d and e, Same as c, except that XPB CFE is replaced by XPC CFE and XPD CFE, respectively, and the TFIIF fractions are indicated (fractions 27-35 were used for XPB and XPD, and fractions 25-34 were used for XPC, based on previous experiments). For the XPD CFE complementation assay, 280 ng TFIIF protein pool were used. Radiolabelled substrate and CFE were prepared as described (refs 12 and 28, respectively). Mutant cell lines used have been described²⁵.

FIG. 4 TFIIH helicase activity is modulated by TFIIIE. **a**, DNA helicase activity was measured by the release of a 34-nucleotide oligomer hybridized to single-stranded M13 DNA. Substrate was prepared as described¹ with modifications. **b**, Results in **a** were quantified on the Biorad GS-250 Molecular Imager and percentage helicase activity plotted against TFIIIE units. **c**, Effect of antibodies directed against both subunits of TFIIIE on TFIIIE-dependent inhibition of TFIIH helicase activity. TFIIIE was preincubated with either affinity-purified p34 antibodies or with protein A-purified p56 antibodies. **d**, TFIIH, ERCC2 and ERCC3 were tested for their ability to bind to a GST fusion protein with TFIIIE-p56 (GST-p56). The (+) lanes show the respective inputs of TFIIH, ERCC2 and ERCC3 to the individual experiments with TFIIIE (GST-p56). GST alone serves as a negative control. The TFIIH blot was probed with p62 antibodies. The slower-migrating band in lane 3 of the TFIIH blot is a nonspecific band which is not retained by GST-p56 or by GST alone. The ERCC2 and ERCC3 blots were probed with antibodies as before.

METHODS. **a**, Helicase assay: 2 pmol single-stranded DNA were annealed to 2 nmol of a 24-nucleotide oligomer extended by Klenow fragment (15 units) in the presence of 50 μ M each dGTP and dATP and 5 mCi [α -³²P]dCTP. After extraction with phenol and chloroform, free nucleotides were removed by gel filtration on Sephadex-G75. The DNA helicase assay (15 μ l) contained 3 mM HEPES-NaOH, pH 7.9, 20 mM Tris-HCl, pH 7.9, 7 mM MgCl₂, 2 mM DTT, 3% glycerol, BSA at 0.5 mg ml⁻¹, 2 mM ATP and 100 ng substrate (20,000 c.p.m.) as described¹. **b**, Results in **a** are plotted as per cent helicase activity versus TFIIIE units; 0.5 μ l TFIIIE typically saturates our transcription assays. Therefore 0.2, 0.4, 0.6 and 0.8 TFIIIE units are equal to 0.2, 0.4, 0.6 and 0.8 μ l purified TFIIIE. **c**, Anti-repression of helicase activity was achieved by preincubating TFIIIE with either anti-p56 or anti-p34 antibodies for 45 min at 4 °C. **d**, GST pull down assay: 200-fold excess



GST-p56 was incubated at 4 °C in the presence of either 10 μ g TFIIH, 100 μ g ERCC3 (*E. coli* with maltose-binding protein (MBP)-ERCC3 fusion), or 100 μ g ERCC2 (MBP-ERCC2 fusion) induced cell-free extract for 1 h. A 50% slurry of GST resin (30 μ l) was then mixed with the proteins and incubated at 4 °C for an additional 45 min with mild agitation. The GST beads were then pelleted at 3,000 r.p.m. for 2 min and washed 5 times with buffer containing 40 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 0.4% NP-40, 1 mM DTT and 0.5 mM PMSF. Bound proteins were separated on a 9% SDS-polyacrylamide gel and analysed by western blotting.

initially identified as nucleotide-excision repair proteins^{2,16,24} before they were implicated in transcription^{10,11}. The question that arises is whether the XPB and XPD proteins perform their repair function as TFIIH, as individual subunits, or as a repair complex containing subunits other than those found in TFIIH. We believe that these proteins perform their function in the form of TFIIH for the following reasons. Contrary to *in vivo* experiments in which cell fusions of XPB (ERCC3) and XPD (ERCC2) mutants yielded full complementation, cell-free extracts from XPB (ERCC3) and XPD (ERCC2) cell lines failed to complement, or gave only marginal complementation in an *in vitro* excision assay²⁵. The most plausible explanation of these results is that in the *in vivo* complementation, functional TFIIH can be assembled with wild-type XPB and XPD, but *in vitro* the TFIIH complexes containing mutant XPB and mutant XPD do not readily exchange subunits, and as a result very little TFIIH with wild-type XPB and XPD exists. Indeed, our attempts to complement the XPD cell-free extract with purified XPD (ERCC2) failed, reflecting the stability of the TFIIH complex and the difficulty of exchanging subunits. We conclude that TFIIH carries out RNA polymerase II transcription and DNA repair as a functional unit. □

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Get the red out with Robbins Scientific's reagent.

when purifying lymphocytes on density gradient media (*Reader Service No. 101*). Human blood is incubated at room temperature for 5 min with Red-Out, then centrifuged in Robbins Scientific's Iso-Prep isolation media or other ficoll-isopaque-type media. The company says that the red cell aggregates that form settle easily to the bottom of the tube. Red-Out is based on a murine monoclonal antibody that binds to a universally present antigen on erythrocyte membranes. It is said to be effective in the removal of red cells, including immature nucleated erythrocytes, which interfere in a number of applications including HLA tissue typing tests, microscopic analysis and fluorescence-activated cell sorting. Two sizes are available — sufficient for 250 or 1,000 tests.

Invitrogen is offering **electroporation cuvettes** in 0.1, 0.2 and 0.4 cm gap sizes for electroporation of bacterial, yeast and mammalian cells (*Reader Service No. 102*). The cuvettes are intended for use with the company's Electroporator II and in electroporation units available from Bio-Rad, BTX and IBI. Gap widths, said to be consistent within a few thousandths of an inch, are designed to provide reproducible results. All cuvettes are gamma-irradiated and colour-coded.

Fisher Scientific's Compac **personal microcentrifuge** is a handy, lightweight unit

that is suitable for a variety of applications, including microfiltration of HPLC samples and cell separations (*Reader Service No. 103*). Users can choose from dual- and variable-speed models, which are both equipped with an insulated motor for quiet, vibration-free operation. The dual-speed Micro D centrifuge can be set to 6,000 r.p.m./2,900 g or 9,000 r.p.m./5,800 g; the variable-speed Micro V to 10,000 r.p.m./7,200 g, in 1,000-r.p.m. increments. Both measure

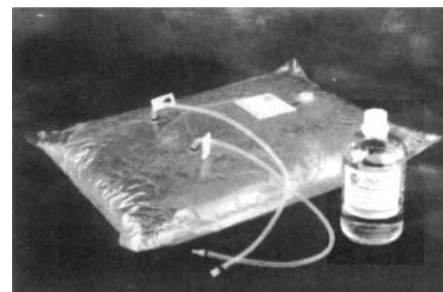


Fisher Scientific's compact personal microcentrifuges come in dual- and variable-speed models.

19 × 18 × 10 cm and weigh less than 2.3 kg. Other design features include: a digital readout that can alternatively display speed and g-force readings during a run, a digital timer that lets the user select a broad range of run times, from 30 s to 90 min, and a lid-locking mechanism that prevents opening of the lid while the rotor is moving. Included with each unit are two 45° fixed-angle polycarbonate rotors: one 8-place rotor for 1.5–2.0-ml tubes and one 16-place rotor for 0.5-ml tubes. (A 16-place rotor for 0.4-ml tubes is also available.) On the safety side, the individual tube slots provide sample containment in the event of tube breakage, and a rotor seal prevents aerosols from escaping from the unit. Rotors and seals are autoclavable.

■ Cell culture

Hybrid Grow from Unisyn Technologies is a specialized **basal medium for growing hybridomas** in Cell-Pharm hollow-fibre reactors (*Reader Service No. 104*). The medium, produced in a Class 100 clean room in compliance with US good manufacturing practices, elevates glucose and glutamine levels to deliver more efficiently nutrients to the tissue-dense hollow-fibre hybridoma cultures. It is designed to simplify down-



Hybridoma growth medium.

stream processing and reduce regulatory concerns because it is free of bovine-derived products. By adding defined medium supplements to enriched Hybrid Grow, the company says that rates of production are increased, even in a serum-free environment. In addition, the medium does not contain any phenol red, which can interfere with charged-based separation media such as ion-exchange gels.

Gel Gro is ICN Biomedicals' **agar replacement gelling agent** (*Reader Service No. 105*). This naturally derived, highly purified polysaccharide is intended as a matrix for the preparation of nutrient media for most microbiological applications and plant



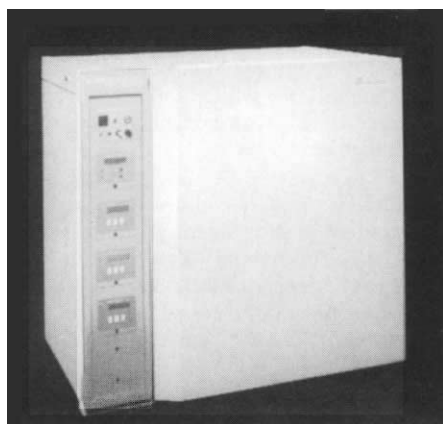
Gel Gro — an agar replacement gelling agent from ICN Biomedicals.

tissue culture work. It is said to hydrate and disperse readily in both hot and cold deionized water (in cold water it forms viscous solutions), yield high gel strengths at low concentrations (approximately one-half the concentration of agar) in the presence of soluble salts, have low viscosity at high temperatures, be able to withstand routine autoclaving and be resistant to enzymatic degradation.

Sigma Immunochemicals offers **human, recombinant keratinocyte growth factor (KGF)**, expressed in *Escherichia coli*

(Reader Service No. 106). Product purity is stated as ≥ 96 per cent by SDS-PAGE and amino acid sequence analysis. It is supplied sterile-filtered and lyophilized at 10 microgram per vial. The company is also making available a line of **cell linker molecules for the stable labelling of cell membranes**. Sigma says that cell membranes labelled *in vitro* can be tracked for up to 100 days *in vivo*. The labels can be followed for up to 10 cell divisions. A green and red fluorescent dye facilitate dual labelling. The cell linkers are said to be non-toxic, stable and have no effect on the biological activity or proliferative activity of cells. They can be detected by either fluorescent microscopy or flow cytometry using standard laboratory equipment. In addition, both red and green fluorescent dyes have been attached to linkers designed particularly for phagocytic cell labelling.

For all your cell culturing needs, Forma Scientific has introduced a HEPA-filtered IR incubator (Reader Service No. 107). The Model 3860 incubator provides 7.4 cubic



Forma Scientific's hepa-filtered incubator.

foot of usable space and features 'touch-pad' controls for ease of operation. The unit is designed to provide continual decontamination via a patented airflow system. The stainless steel interior is smooth seam welded, with no crevices, for further prevention of contamination.

Immunology

Amersham offers a new enzyme-based scintillation proximity assay (SPA) designed to assist researchers working in antiviral drug screening programmes. The assay is intended specifically to **identify inhibitors of helicase from the herpes simplex virus (HSV)** (Reader Service No. 108). (Helicase is responsible for the unwinding of double-stranded DNA prior to replication, and is therefore essential to the infective nature of HSV and related viruses. Consequently, this enzyme serves as an important therapeutic target for antiviral drugs.) The assay may, however, be useful in studies involving other viruses from the herpes family or other double-stranded DNA viruses, for example, the adenoviruses and poxviruses. It can be per-

formed in a single tube. The kit, which excludes the enzyme and inhibitor samples, contains sufficient reagents for 500 assays.

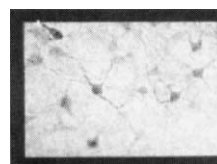
Researchers using recombinant DNA technology can exploit the **epitope tagging strategy** for screening colonies, localization and purification of expressed proteins and quantification by immunoprecipitation. This strategy relies on the availability of specific antibodies directed against unique peptide epitopes. The Berkeley Antibody Company offers three families of such antibodies: polyclonal antibody HA11, which recognizes an influenza haemagglutinin epitope, monoclonal antibodies AU1 and AU5, which are specific for sequences of the bovine papilloma virus, and the monoclonal antibody 9E10, which is specific for a portion of the human *c-myc* gene product (Reader Service No. 109).

Transcription products of Ambion's new **oncogene probe templates** can be used as probes in ribonuclease protection assays, *in situ* hybridization experiments and northern blots to detect the presence of oncogene mRNA (Reader Service No. 110). These templates contain fragments of oncogenes produced from cDNA. Each fragment has been designed to span at least one intron-exon boundary. For each template insert, Ambion has chosen a region of the oncogenes which does not have high homology to related genes (for example, pseudogenes or members of multigene families), is not a region of known mutational hotspots (such as rearrangements or point mutations), and is not involved in alternative splicing. The sizes of the protected fragments produced by these templates have also been designed so that these probes can be used simultaneously in multiple probe experiments. The line-up includes: pTRI-*c-myc*-human, pTRI-*erbB2*-human, pTRI-p53-human, pTRI-IGFR-human, pTRI-EFGR-human, and pTRI-MDRI-human.

Oncogene Science is offering **antibodies to markers of metastasis** such as urokinase plasminogen activator (uPA), cathepsins (B, D and L), bFGF and nuclear matrix proteins such as lamin B (Reader Service No. 111). Three monoclonal antibodies to uPA are available: two recognize the B-chain by immunoblotting, immunoprecipitation and immunohistochemistry, while a third recognizes the A-chain by immunoprecipitation. An ELISA and a DNA probe are also available. Eight monoclonal and polyclonal antibodies are available for cathepsins B, D and L that react with epitopes in the pro-regions and with the mature form. The antibodies may be used for immunoblotting, immunoprecipitation and staining of frozen and paraffin-embedded tissue sections. A competing peptide for each cathepsin and an ELISA for cathepsin D are also available. Five antibodies to bFGF are provided for immunoblotting, immunoprecipi-

tation, ELISA and immunohistochemical analysis. Two bFGF peptides and specific probes for bFGF and bFGF receptor are provided. Completing the line-up are five antibodies to nuclear matrix proteins, including lamin B, used in immunofluorescence analysis, staining of frozen sections and immunoblotting.

Chemicon's new **rabbit polyclonal to NMDAR1** is selective for the splice forms NR1-1 and NR1-2 (Reader Service No. 112). The antibody is affinity purified and may be used for immunocytochemistry (both light and electron microscopy), western blotting and immunoprecipitation. The NMDA receptor plays a key role in the central nervous



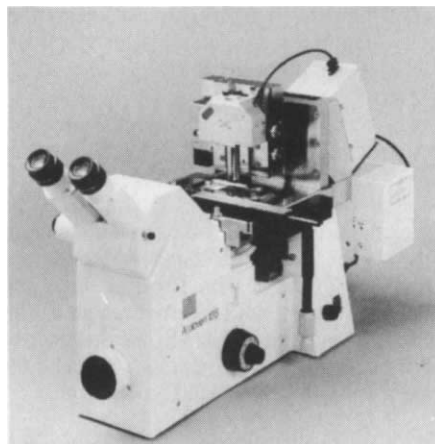
Polyclonal to ADA.

system and has been shown to be directly involved in neuronal development, synaptic plasticity, and learning and memory. It is also involved in neuronal degeneration and neuron death, and has been implicated in several neurodegenerative diseases. Also of interest is a new **rabbit polyclonal antibody to adenosine deaminase (ADA)**, which can be used for immunocytochemistry (both light and electron microscopy) and western blotting. Chemicon says that it has been used successfully to detect ADA immunoreactive neuron populations in rat central nervous system.

AMAC's MRK-16 **antibody recognizes a surface epitope of human p-glycoprotein**, a 170-kD human multi-drug resistant product, which acts as an efflux pump to remove toxic products from the cell (Reader Service No. 113). The antibody may be used to investigate haematological malignancies by flow cytometry and immunohistochemical staining of solid tumours. It is provided as a liquid concentrate.

Small world solutions

The new BioScope **scanning probe microscope** from Digital Instruments can be used for imaging cells and tissues *in situ* with nanometre resolution, the company says



BioScope — nanometre resolution.

(Reader Service No. 114). Designed to meet the needs of biologists and life science researchers, the BioScope combines fluorescence, confocal laser or other optical techniques with atomic force microscopy. A magnification range of $\times 25$ to $\times 10,000,000$ is provided. Samples can be imaged dry or in fluid, allowing *in situ* examination of cells and tissues on slides or Petri dishes. The system incorporates a modified Zeiss inverted optical microscope, which views the sample from below while the atomic force microscope images the sample from above. The company also offers the NanoScope Dimension 3000 atomic force microscope, designed to support most AFM/STM scanning techniques. With the Dimension 3000, researchers can analyse samples up to 8 inches in diameter in air or liquid with manual



Digital Instruments — taking a new dimension with its NanoScope Dimension 3000 which handles samples up to 8 inches in diameter.

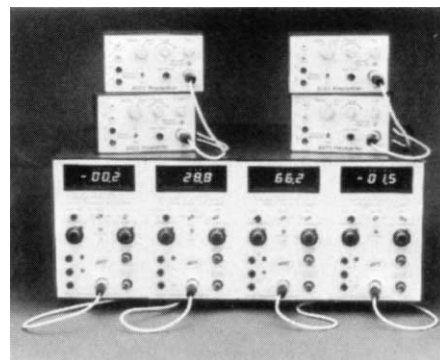
and automated stepping for scanning multiple areas of the sample completely unattended. Other design features include built-in top-view video optics with motorized zoom and the TrakScan laser tracking system. Digital Instruments says that the microscope offers good resolution and linearity in all three dimensions and is particularly suited to magnetic media and semiconductor applications. Vacuum accessories are standard equipment for mounting wafers, disks and other samples.

Meridian Instruments has introduced a Pentium-based version of its Insight-IQ image processing and quantitation computer system (Reader Service No. 115). Available as an option with the company's Insight Plus laser scanning confocal microscope, the Insight-IQ system can be supplied in a standard 80486 DX2/66 MHz configuration, or with the Pentium-based microprocessor for enhanced speed and performance. Dedicated software provides access to a variety of operations involving scanning, image processing, image optimization, display optimization, image analysis, kinetics analysis, two- and three-dimensional renderings, and systems management. Also available is the Ultima Premium laser confocal microscope system which couples a UV-visible laser with two independent,

optically corrected lightpaths to provide simultaneous excitation of the UV line and one of the two visible lines. Its dual AOMs and 66 MHz Pentium-based computer system can switch between UV and visible excitation lines in as little as 4 ms, the company says. Stated applications include the release and tracking of caged compounds, the generation of two- and three-dimensional confocal images, the simultaneous excitation and detection of UV and visible fluorochromes, the imaging and quantitation of calcium (or other ions) and pH over time or through *x*, *y* or *z* planes, and the scanning of areas as large as 2×2 cm and the generation of images as large as $1,536 \times 1,536$ pixels.

■ Voltage clamps

The EVC-4000, a voltage and current clamp from World Precision Instruments, allows clamping of up to four epithelial membranes simultaneously (Reader Service No. 116). The mini-perfusion system and a luer-tipped cartridge electrode are said to reduce set-up time, allow simultaneous testing of multiple living membranes and reduce the voltage levels needed to control



WPI's EVC-4000 multi-channel voltage clamp allows voltage clamping of up to four epithelial membranes simultaneously.

them. The device is modular, housing from one to four amplifiers. Each module, with its companion preamplifier and potentiostat, operates independently, actively maintaining one surface of the test membrane at zero potential relative to ground under all operating conditions. Each operates as voltage clamp, current clamp, or open circuit potential.

Two new electrode voltage clamps for electrophysiology, the VC-1200 two-electrode voltage clamp and the VC-3100 single-electrode version, are now available from Medical Systems (Reader Service No. 117). Two-electrode voltage clamping can be used for studying electrical events occurring in cell membranes: one electrode is used to record membrane potentials, the other passes the clamping current. The VC-1200 has been designed with a wide current and voltage capacity. It has speed, amplification and the flexibility needed to clamp small cells, while having the voltage com-

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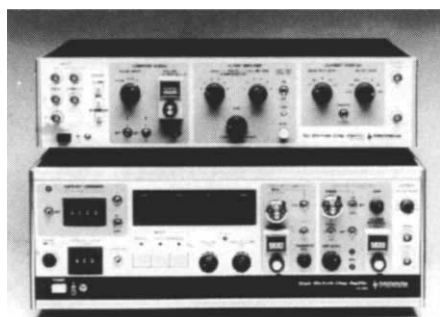
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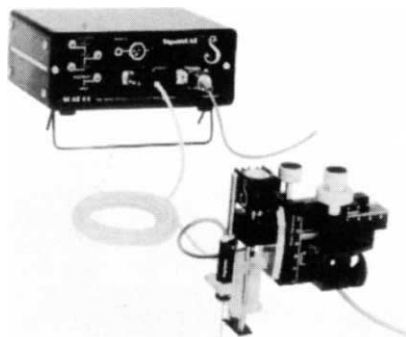
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Reader Service No.15



Electrode voltage clamps for electrophysiology — see Medical Systems.

pliance (± 130 V) successfully to clamp large cells, such as frog oocytes. For those on a budget, the company offers the VC-1200 unit, which includes an automatic overload cut-out to protect cells, stimulus mode for use with iontophoretic stimulation, two pole adjustable bandpass filter, virtual/chassis ground options, and phase controls. Single electrode voltage clamping can be used in the study of electrical events occurring in small cell membranes. The VC-3100 offers three modes of operation: bridge current clamp, sampling voltage clamp and sampling current clamp. It is designed to distinguish between actual membrane potentials and artifacts, and features a miniature low noise headstage with up to 10 pF of capacitance compensation. Outputs and inputs for interfacing with computers (including mode switching) are available on the rear panel.



Scat-01 Ultra-Stepper — new on the market for neurophysiologists.

Also offered is the Significat Scat-01 Ultra-Stepper, a computer-controlled microelectrode stepper system for use in neurophysiological experiments. The Scat-01 is said to provide movement fast enough to avoid 'dimpling' in brain tissue and to penetrate many small cells. Common step patterns are available with the software included; alternative patterns can be developed by the user using the BASIC computer language supplied. An ultrasonically-coupled keypad allows for remote control.

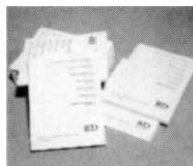
■ Catalogues and guides

Promega now offers a line of **signal transduction products** for studying protein phosphorylation and the many pathways that control eukaryotic transcription (*Reader*

Service No. 118). The line-up includes purified kinases, highly specific purified kinase peptide substrates and inhibitors, antibodies, and quantitative assays for measuring kinase activity. The signal transduction product line is designed to complement Promega's existing line of cytokines, growth factors and eukaryotic transcription factors and provide the researcher with a full range of products for studying cellular signalling.

Pierce has released its all-new **catalogue and handbook** which features a 350-page technical section and a 230-page ordering section (*Reader Service No. 119*). The technical section provides researchers with information about techniques like antibody production, antibody fragmentation, cross-linking/protein modification, clinical and research assays, electrophoresis, enzymatic labelling, gas chromatography, HPLC, immunoassays, iodination, protein assays and protein immobilization.

R & D Systems has a new 1994 **catalogue** containing many products useful to researchers working in immunology, molecular and cellular biology (*Reader Service No. 120*). The catalogue is split into five major sections — cytokine proteins and antibodies, adhesion molecule research products, Quantikine cytokine immunoassays, flow cytometry reagents, probes and genes — and also features a review of the scientific literature.



R & D Systems' current catalogue.

A detailed **monoclonal antibody purification guide**, which focuses on the detection, purification and measurement of monoclonal antibodies, is now available from Sterogene Bioseparations (*Reader Service No. 121*). It describes methods to purify monoclonal antibodies from ascites fluid or cell culture, even in the presence of high concentrations of bovine IgG. Details on how to use the company's non-ELISA-based antibody measurement kit, a kit to qualify mouse ascites or culture supernatant, and an antigen coupling kit for antibody purification are also included.

■ Molecular biology

The Foto/Force 150 **electrophoresis power supply** from Fotodyne is designed to serve the needs of both educators and researchers alike (*Reader Service No. 122*). The unit provides variable voltage from 10 to 150 V at up to 300 mA. Safety features include overcurrent, short circuit and arc-over protection. Up to two electrophoresis chambers may be run with the standard unit, and up to eight with the optional PowerCube module.

Pharmacia Biotech announces the new GeneQuant II **RNA/DNA calculator**, pro-

viding instantaneous results to help researchers monitor the progress of essential steps in mapping, cloning and sequencing (*Reader Service No. 123*). The instrument uses measurements with nearest neighbour base sequencing data. Researchers just key in the ACGT or ACGU base sequencing (up to 66mer) to obtain instant calculation of oligonucleotide conversion factor ($\mu\text{g ml}^{-1}$), molecular weight, theoretical absorbance ($\text{AU } \mu\text{mol}^{-1}$) and theoretical T_m . The instrument provides automatic DNA/RNA and oligonucleotide quantification with (A_{260}/A_{280}) purity check.

Hitachi Software, in conjunction with Molecular Probes, has developed four new **reagent kits** for use with its FMBIO-100, filmless, nonradioactive fluorescent-method image analysis system (*Reader Service No. 124*). Kits for nucleic acid staining, western blotting, tangerine oligonucleotide labelling and tangerine fast CAT (chloramphenicol acetyltransferase) activity are available.

The Discrete-Delete kit from Epicentre Technologies is designed to **produce unidirectional sets of nested deletions** in double-stranded DNA targets (*Reader Service No. 125*). Resulting deletion clones can be used in functional and domain mapping of genes and gene products, dideoxy sequencing and other applications. The method takes advantage of the predictable digestion rate of exonuclease III and the strict specificity of mung bean nuclease for single-stranded DNA to produce uniform, reproducible rates of deletion. Digestions proceed in a 5' to 3' direction from a blunt end or 5' overhang. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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Your future in your hands

David P. Collingham

While the traditional career ladder may remain, for many the career pathway — the means of achieving one's career objectives — has altered significantly.

LATE last year the head of a large R&D department of one of the most successful biotechnology organizations walked into our offices for a career guidance discussion. He had been recruited previously from a secure R&D management position in a major international pharmaceutical company. Things had been going well, but all of a sudden he was confronted with an organizational change which left him no longer head of department. Indeed he had a new manager, recruited externally, put in over him. He was far from happy.

This scenario is common in emerging companies — the environment for an individual's career development, very appropriate in one corporate culture, is inappropriate in the next. It seems that the fabric of career management in corporate R&D is changing significantly, as organizations strive to adapt to the evolving demands of the marketplace and the ambitions or expectations of employees.

Traditional career ladder

The traditional view has it that as a scientist you enter at the bench level and progress periodically, to the point at which you regard yourself as a 'manager'. If you have what it takes, and are lucky, you may be rewarded by a functional transfer and further promotion. If not, you languish at or near the bench performing the same old routines, with more paperwork but not responsibility.

What makes the transition from scientist to manager? Again, the traditional view has it that you will be rewarded with increased responsibility, managing greater resources, staff, equipment and capital expenditure, and allowed greater decision-making in research strategy.

According to Chris Ainsworth, Development and Training Advisor at Zeneca Pharmaceuticals R&D headquarters, there are many pitfalls in treading the pathway from science to management. He likens the career ladder to a train journey with fixed entry and exit points, and a known beginning and end. The career pathway is by contrast a flexible, dynamic, self-adjusted process, like a motorway journey with options and possibilities for modification. The employing organization provides the infrastructure for these decisions, but the onus for the decisions themselves is on the individual employee.

Contemporary model

Zeneca is going through a process of



change, with an increasing recognition of the 'contemporary' model for career development, including cross-functional change. For example, Ainsworth cites the combination of project management and therapeutic portfolio management, as opposed to rigid adherence to functional organization as chemistry, biology, pharmacology, and so on. Today, Zeneca's Discovery Research is freshly organized into Therapeutic Area teams, each with its full complement of functional specialities.

But not everyone wants to be a manager. What about the good scientist who wants to remain a scientist? Companies are increasingly allowing for this, and rewarding scientific endeavour. Zeneca and its former parent ICI adopt the 'scientific-ladder' principle which does just that, recognizing scientific value and rewarding the scientist for doing what he or she is best at.

So what does the 'contemporary' career pathway model show? Broadly speaking, organizations are breaking down the traditional, functional barriers. Jobs are becoming more skills-based, with an emphasis on seeking to optimize an individual's specific skills. This view is reinforced by Alan Garmonsway, Head of Human Resources at Xenova in the United Kingdom. Looking from the corporate viewpoint, Garmonsway tries to align the individual's strengths and preferences

with the specific role to fulfil the objectives of both the employer and employee. "If you operate on a skills base, there is the greater possibility of modifying the job around the key skills of the individual." In his recent experience with the rapidly growing Xenova drug discovery team, this means that recruitment and staff development occurs organically rather than in the traditional linear manner.

Disappearing ladders?

According to research carried out by Inkson and Coe¹, the global recession has had a major impact on career management. From a sample of more than 800 managers, they reported that lateral career changes are increasing and upward moves declining.

Within the biosciences, our experience reinforces these observations. Certainly, career management in the United States and Europe has changed greatly in the past few years, with 1992 and 1993 being seen as a critical period. We used the phrase 'career management' — to be specific, we should add the word 'active', for there is only one person who can steer this process — the individual scientist.

We find, from our experience of recruiting managers in the global biotechnology and biosciences industries, that scientists can be extraordinarily inward-looking. They ought to be better able to analyse and admit their own competencies, and be prepared to listen to others.

The pathway from bench scientist to senior manager is often a long and tortuous one, suited only to a tiny minority of individuals who happen to possess the key ingredients for success at a number of stages on the career ladder. They have come to terms with the loss of so many of the fundamental practices of the scientist, to be adaptable and to adopt new ways of thinking and acting. The variables we see in this pathway are numerous and only a few survive it. "People are preconditioned to expect periodic career advancement through promotion", says Peter Nicholls, director of personnel at Celltech, "but we should be encouraging an atmosphere of self-advancement within the role".

Nicholls has seen Celltech grow to its current staffing level of 410. As the company matures, so do its staff. Average age has risen over the past six years (from 29 to 33). Labour turnover is low and staff are actively encouraged to develop new skills within their existing roles. Nicholls sees a

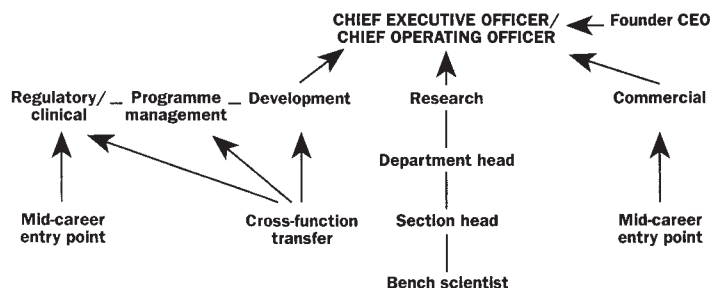
far more subtle form of career progression, involving active career management, linked to an understanding of the dynamics of the growing corporate animal. For example, if people stayed basically in the same job, they would find the dimensions of the job changing, becoming larger, more demanding and different. It follows that the individual needs to respond positively to these dynamics, despite the fact that their job title may not change. The outcome of this is a new kind of career development based on broadening skills, which thereby improves the individual's worth and marketability.

We observe some inability of scientists to adapt themselves to the changing needs of the marketplace, leading to career frustrations in ambitious PhDs and graduates both in Europe and the United States. One topical point which crops up regularly is the difficulty in allowing non-PhD graduates to realize their full potential within the scientific work place. By and large PhDs find cross-functional transfer easier, and in many companies entry to the senior management club is usual only for PhD holders. We see differences of opinion here within different companies on both sides of the Atlantic, with a growing realization that people should be assessed on a competence basis rather than on sheer knowledge or experience. In the United States we see a more

Darwinian approach to peer competition; factors such as visibility to top management, and managing relationships with superiors, become important prerequisites for career advancement.

So for those scientists seeking career advancement in the 1990s, here are a few key points drawn from our global recruitment experience:

- Manage your career — don't let others do it for you. Work out your likes/dislikes and seek out roles which give positive reinforcement.
- Develop your career plan; know your start point and map out the way points; mould your career to fit your life goals.
- Discuss your strengths and weaknesses with others. Communicate your preferences. Seek external advice.
- Your real capital is your own experience; it's yours, not your employer's.



The paths to the top.

- Seek advancement but do not necessarily associate this with a change of job or role, although a change of organization/division may be a catalyst to further advancement.
- Learn to realize your limits; equally learn to know when you are on top of the job and need new horizons.
- Manage relationships with your superiors, increase your visibility and seize every opportunity to broaden your experience.

David P. Collingham is chairman of Ruston Poole International, founder member of Global Partners, a worldwide executive search network specializing in healthcare and biosciences. Ruston Poole International is based at 11/12 Buckingham Gate, London, SW1E 6LB, UK.

1. Inkson, K. & Coe, T. *Institute of Management Report* (London, 1993).
2. Collingham, D. P. *Bio/Technology*, 11, S44 (1993).

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Categories of paper

Review Articles survey recent developments in a field. Most are commissioned but suggestions are welcome in the form of a one-page synopsis addressed to the Reviews Coordinator. Length is negotiable in advance.

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Articles are research reports whose conclusions are of general interest and which are sufficiently rounded to be a substantial advance in understanding. They should not have more than 3,000 words of text (not including figure legends) or more than six display items and should not occupy more than five pages of *Nature*.

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Letters to Nature are short reports of outstanding novel findings whose implications are general and important enough to be of interest to those outside the field. Letters should have 1,000 or fewer words of text and four or fewer display items. The first paragraph describes, in not more than 150 words and without the use of abbreviations, the background, rationale and chief conclusions of the study for the particular benefit of non-specialist readers. Letters do not have subheadings and contain fewer than 30 references.

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Scientific Correspondence is for discussion of topical scientific matters, including those published in *Nature*, and for miscellaneous contributions. Priority is given to letters of fewer than 500 words.

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Titles are brief and simple. Active verbs, numerical values, abbreviations and punctuation are to be avoided. Titles should contain one or two key words for indexing purposes.

Artwork should be marked individually and clearly with the author's name and, when known, the manuscript number. Ideally, no figure should be larger than 28 by 22 cm. Figures with several parts are to be avoided and are permitted only if the parts are closely related, either experimentally or logically. Unlettered originals of photographs should be provided. Suggestions for cover illustrations, with captions and labelled with the manuscript number, are welcome. Original artwork is returned when a manuscript cannot be published.

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Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided whenever possible and, if used, defined. Footnotes are not used in the text.

Acknowledgements are brief and appear after the reference list; grant and contribution numbers are not allowed.

Supplementary information is material relevant to Articles or Letters which cannot, for lack of space, be published in full, but which is available from *Nature* on request.

Submission. Manuscripts can be sent to the Editor at 4 Little Essex Street, London WC2R 3LF, UK or at 1234 National Press Building, Washington, DC 20045, USA. A telephone and fax number should be included. Manuscripts or proofs sent by air courier to London should be declared as 'manuscripts' and 'value \$5' to prevent the imposition of import duty and value-added tax.